REMARKS

Claims 6-11 are in the case. These claims are finally rejected on 112 grounds. There are no prior art rejections.

There are two 112 rejections.

We turn to the first of these 112 rejections. This rejection is based on the written description requirement. The Office Action at page 2 takes the position that there is no support for the claims at page 11 of the specification as asserted.

Reconsideration is requested.

Below are quoted the portions of page 11 of the specification on which applicant is relying.

> Passing of one or more screening tests of the first embodiment of the invention herein maximizes the opportunity of the agent passing the test, being successful for the treatment of and in the second embodiment herein. The more of the tests (a), (b), (c), (d), (e), (f) and (g) passed, the greater the likelihood of success.
>
> We turn now to the second embodiment of the invention herein which is directed at a method for treating a patient having or at risk for cancer, Alzheimer's disease or atherosclerosis, comprising administering a therapeutically effective amount of a selective inhibitor or COX-2 that meets at least one of, preferably at least two of, (a), (b), (c), (d), (e), (f) and (g).

It is submitted that the above provides the support the Office Action says is missing.

We turn now to the second of the 112 rejections. This rejection is based on the enablement requirement.

At one place or another in the Office Action, the following positions seem to be asserted.

(1) In vitro screening tests have no utility in respect to developing drugs for cancer treatment (relying on Gura and Johnson);

(2) The claims are directed to screening for "likelihood of success" in treating disease and likelihood of success is not defined;

(3) The tests recited in the claims are not sufficient for developing drugs for treating all cancers;

(4) The tests of claim 6 are not enabled for likelihood of success in treating Alzheimer's disease (AD) because AD cannot be diagnosed without autopsy and because evaluation of treatment results is not possible because of variation of progression of disease from patient to patient (relying on MedicalNet.com);

(5) Data obtained from animal models in development of drugs for treating Alzheimer's disease is not conclusive (relying on Firuzi);

(6) The drugs to which the testing of the claims is directed are counterindicated for treating or preventing atherosclerosis because they pose cardiovascular risk.

Reconsideration is requested.

In regard to points (1) and (2), empirical testing of cancer drugs on patients without lead up testing is not how drug development works and would not be allowed by the FDA. Rather there is screening in vitro to provide a rationale followed by animal studies, followed by a series of human studies, followed by clinical testing. The term "likelihood of success" does not need to be defined because those skilled in the art would understand that this means selection of drugs for testing in the next step, animal studies, that is to provide a baseline for study of a drug leading to FDA approval. One has to start somewhere in evaluating drugs for

cancer treatment. The instant tests would be considered by those skilled in the art, as the starting point.

Furthermore, literature shows that the normal progression from in vitro screening to animal studies to human clinical studies is what occurs in development of cancer drugs.

In this regard, see Steinbach, G., et al., The New England Journal of Medicine 342(26), 1946-1952 (2000), copy enclosed, which shows progression from preclinical studies to clinical studies for COX-2 inhibitors for treating colon neoplasia (cf to claim 7).

See also Swain, S., The New England Journal of Medicine 353(26), 2807-2809 (2005) and Voskoglon-Nomitos, T., et al., Clinical Cancer Research (9), 4227-4239 92003), copy of each enclosed, which show predictive value for in vitro cell culture, animal models, mouse allograft preclinical and human xenograft preclinical studies, in predicting successful clinical results.

In respect to (3) above, over thirty types of cancers and precancerous conditions to which the invention applies are listed at page 11, last nine lines, to page 12, first four lines, of the application as filed. This should be enough for reciting cancer generally in claim 6.

In respect to (4) above, it is noted that Alzheimer's disease is routinely diagnosed without autopsy (see page 1397 or and 1398 of the Merck Manual (17 edition), copy enclosed. Tens of thousands are diagnosed as being affected with Alzheimer's each year. Page 1398 of the Merck Manual indicates that effectiveness of treatment can be determined.

In respect to (5), the claims do not involve animal models but are a baseline for progressing to further testing. Moreover, whether or not animal models may or may not provide conclusive data, is irrelevant, since animal testing would be followed by clinical testing.

In respect to (6), it is noted that drug safety is an issue for the FDA and not the PTO. See Scott v. Finney, 32 U.S.P.Q.2d 115 (Fed. Cir. 1994), In re Anthony 162 U.S.P.Q. 594-604 (CCPA 1969), and Kandic v. Ragunothau, 73 U.S.P.Q.2d 1180 (Bd. Pat. App. & Int. 2001). Note that the FDA allows and has not banned chronic administration of selective inhibitor of COX-2. Risks are currently accommodated for by box warnings and the decision on risk is left to a patient's physician after a risk/benefit analysis. The PTO should not interfere with this current scenario.

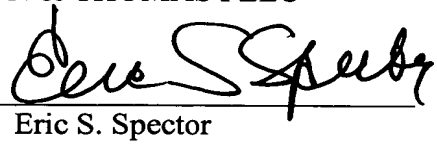
Note that inflammation contributes to each of the diseases mentioned in the claims and the tests of claim 6 are known to be directed to inflammatory activities. These tests indicate whether a potential drug may provide anti-inflammatory benefit by route in addition to COX-2 inhibition.

See also Collins, A.R., Arterioscler. Thromb. Vacs. Bio., (March 2001), 365-371, which indicates the appropriateness of test (a) of claim 6 for screening for drugs for atherosclerosis treatment or prevention.

Consideration of this response and its enclosures and allowance are requested.

Respectfully submitted,

BACON & THOMAS PLLC

By: 

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Case No: CRF D-2756
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Whitehouse Station, N.J. 1999

TABLE 171–5. DIAGNOSTIC CRITERIA FOR DEMENTIA

Development of multiple cognitive deficits manifested by both:

1. Memory impairment (impaired ability to learn new information or to recall previously learned information)
2. One (or more) of the following cognitive disturbances:
   a. Aphasia (language disturbance)
   b. Apraxia (impaired ability to carry out motor activities despite intact motor function)
   c. Agnosia (failure to recognize or identify objects despite intact sensory function)
   d. Disturbance in executive functioning (ie, planning, organizing, sequencing, abstracting).

Each of the cognitive deficits described above causes significant impairment of social or occupational functioning and represents a significant decline from a previous level of functioning.

The course is characterized by gradual onset and continuing cognitive decline.

Deficits do not occur exclusively during the course of delirium.

For Alzheimer's disease:

The cognitive deficits listed in the first criterion (parts 1 and 2) are not due to any of the following:

1. Other CNS conditions that cause progressive deficits in memory and cognition (eg, cerebrovascular disease, Parkinson's disease, Huntington's disease, subdural hematoma, normal-pressure hydrocephalus, brain tumor)
2. Systemic conditions known to cause dementia (eg, hypothyroidism, vitamin B_{12} or folic acid deficiency, niacin deficiency, hypercalcemia, neurosyphilis, HIV infection)
3. Substance-induced conditions.

For vascular dementia:

Focal neurologic signs and symptoms (eg, exaggeration of deep tendon reflexes, extensor plantar response, pseudobulbar palsy, gait abnormalities, weakness of an extremity) or laboratory evidence indicates cerebrovascular disease (eg, multiple infarctions affecting the cortex and underlying white matter) that is judged to be etiologically related to the disturbance.

For dementia due to other medical conditions:

Evidence from the history, physical examination, or laboratory tests indicates that the disturbance is the direct physiologic consequence of such conditions as Parkinson's disease, Huntington's disease, Pick's disease, Creutzfeldt-Jakob disease, head trauma, HIV infection, normal-pressure hydrocephalus, hypothyroidism, brain tumor, vitamin B_{12} deficiency, or intracranial radiation.

Modified from American Psychiatric Association: *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition. Washington, DC, American Psychiatric Association, 1994, pp. 142–143, 146, 152; reprinted with permission. Copyright, 1994 American Psychiatric Association.

age. They tend to learn new information slowly; if they are given extra time for such tasks, their intellectual performance is usually adequate.

Dementia of depression (formerly termed pseudodementia) is usually used to describe patients who may appear demented at first but have depression rather than a neuropathologic disorder. They regain mental competence when depression is treated. More commonly, depression and dementia coexist; in such cases, treating depression is still important but does not fully restore cognition.

The diagnosis of dementia is based on a thorough history and mental status examination. Diagnostic criteria are listed in TABLE

members. Depressed patients can be distinguished because they eat little, are constipated, sleep less than normal, and behave best at night. They respond slowly but often accurately; they may be semimute, but very few are aphasic. They rarely forget major current events or matters of great personal importance. Severely depressed patients tend to complain of memory loss disproportionate to their examination results. In contrast, patients with dementia seldom complain of memory problems. In contrast to demented patients, depressed patients have unremarkable neurologic examinations.

Prognosis and Treatment

The progression rate of dementia varies

injury due to trauma or transient asystole. Abstention from alcohol by patients with alcoholic dementia can lead to substantial long-term improvement. Controlling hypertension or diabetes may slow or arrest the progression of vascular (multi-infarct) dementia, resulting in improvement in a few patients.

Even when intellectual function cannot be restored or its decline arrested, simple supportive measures (eg, frequent orientation reinforcement; a bright, cheerful, familiar environment; a minimum of new stimulation; regular low-stress activities) can help greatly. Orientation to time is helped by using large calendars and clocks and routinizing daily activities; orientation to person is helped by medical staff members wearing large name tags and repeatedly introducing themselves. The patient requires time to adjust to and become familiar with new surroundings, routines, and people. Explanations should be precise and simple, and nonessential procedures omitted.

Quiet, dark private rooms should be avoided. The room should be reasonably bright and contain sensory stimuli, such as a night-light and a radio or television, to help the patient remain oriented and to focus his attention. The environment should also be safe and secure; for example, signal systems can be installed to monitor those who tend to wander. Overstimulation and understimulation should be avoided. Familiar people and frequent visits by staff members encourage the patient to remain social; isolation should be avoided. Staff members should avoid confronting or physically intimidating the patient. The patient should remain as active as possible; families should include him in activities but avoid activities that cause anxiety or confusion. Exercise to reduce restlessness, improve balance, and maintain cardiovascular tone should be performed daily. Occupational and music therapy helps maintain fine motor control and provides nonverbal stimulation. Group therapy (reminiscence therapy and socialization activities) may help conversational and interpersonal skills, and family counseling can teach family members how to prevent the patient from falling and avoid being hit by him during periods of agitation. If daily routines can be simplified and the caregivers' expectations

patient may actually show some improvement.

Functioning can often be further improved by eliminating or strictly limiting drugs with CNS activity. The optimal use of psychoactive drugs in the elderly to control unwanted behaviors is controversial. However, antidepressants can temporarily improve function in patients who develop clinical depression. Depression should be treated with nonanticholinergic antidepressants, and anxiety and sleep disorders may be treated with judicious doses of short- or medium-acting benzodiazepines. Other behaviors are more problematic. Antipsychotic drugs are commonly used, but their effectiveness has not been established except in psychotic patients. Toxicity occurs frequently and can be severe. If used at all, doses should be kept very small, and they should not be used for long periods. There is no evidence that cholinergic-enhancing drugs benefit patients with non-Alzheimer's dementias.

After the medical evaluation is completed and a course of treatment is established, most of the responsibility falls on the family. Although a cure is rarely available, the clinician can still help the family, eg, by helping them understand that although the disease is progressive in nature, many complicating factors can be controlled. The stresses of caring for a person with dementia are tremendous and can adversely affect the physical and emotional health of family members, compromising care. The clinician can recognize the early symptoms of caregiver burnout and guide families to the appropriate social agencies, thereby enhancing the patient's overall care. Team members (social worker, nutritionist, nurse, home health aide, and others) can assist in providing counseling and support to patients and their caregivers.

The patient's wishes about care should be clarified before he is incapacitated. Financial and legal arrangements (eg, durable power of attorney, durable power of attorney for health care) should be made in the early stage of the disease.

ALZHEIMER'S DISEASE

A progressive, inexorable loss of cognitive function associated with an excessive number of senile plaques in the cerebral

which also contains β-amyloid and neurofibrillary tangles consisting of tau protein.

Epidemiology

Early-onset forms account for only 2 to 7% of cases and are usually due to an inherited genetic mutation. The common form affects persons > 60 yr old, and its incidence increases as age advances.

Four million Americans have Alzheimer's disease, at an annual cost of about $90 billion, including medical and nursing home care, social services, lost productivity, and early death. The disease is about twice as common in women as in men (perhaps because women live longer, but female sex may be a risk factor). It accounts for > 65% of the dementias in the elderly. Vascular dementia and Alzheimer's disease coexist in about 15% of cases.

Etiology

The cause of Alzheimer's disease is not known. The disease runs in families in about 15 to 20% of cases. The remaining, so-called sporadic cases have some genetic determinants. At least four distinct genes, located on chromosomes 1, 14, 19, and 21, influence initiation and progression. Chromosome 21 generates the precursor protein for the amyloid protein, which accumulates in the brain of patients with Alzheimer's disease (as well as with other conditions). Chromosome 19 generates apolipoprotein (apo) E alleles 1 to 4 (ε1 to ε4). The presence of the ε4 allele increases the risk for Alzheimer's disease in whites; ε2 and ε4 alleles increase the risk in blacks. Trisomy 21 produces early Alzheimer's disease in persons with Down syndrome. These findings support the epidemiologic observation that the disease has an autosomal dominant genetic pattern in most early-onset and some late-onset cases but a variable late-life penetrance. Environmental factors are the focus of active investigation. Unproven speculations include low hormone levels and exposure to metals.

Pathogenesis

Neurons are lost within the cerebral cortex, hippocampus, and subcortical structures (including selective cell loss in the nucleus basalis of Meynert), locus caeruleus, and nucleus raphae dorsalis. Cerebral glucose use and perfusion is reduced in some areas of the brain (parietal lobe and temporal cortices in early-stage disease, prefrontal cortex in late-stage disease), as determined by positron emission tomography; whether this reduction precedes or follows cell death is not known. The microvasculature may also be affected, as seen in congophilic angiopathy.

Neuritic or senile plaques (composed of neurites, astrocytes, and glial cells around an amyloid core) and neurofibrillary tangles (composed of paired helical filaments) play a role in the pathogenesis of Alzheimer's disease. Senile plaques and neurofibrillary tangles occur with normal aging, but they are much more prevalent in persons with Alzheimer's disease.

Specific protein abnormalities occur in Alzheimer's disease. β-amyloid protein is thought to contribute to the pathogenesis of the disease. Ongoing research is trying to determine if amyloid is a toxic cause of cognitive decline or a biologic reaction and secondary phenomenon. Apo E proteins, produced in the brain and liver, influence a number of cerebral processes, including amyloid deposition, cytoskeletal integrity, and efficiency of neuronal repair. Apo E's role in Alzheimer's disease is becoming more certain. The protein has three allelic forms called ε2, ε3, and ε4, resulting in six genotypes: ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, and ε4/ε4. The risk of Alzheimer's disease is substantially increased in persons with two ε4 alleles, who are more likely to develop the disease between the ages of 60 and 75 yr. Incidence may be decreased in those who have the ε2 allele. Because about 40% of persons who reach age 85 yr develop some form of diagnosable dementia regardless of apo E status, this genetic test is not very useful in predicting whether a person will develop Alzheimer's disease later in life. The test is commercially available. Its usefulness as an adjunctive diagnostic test (rather than a predictive test) for Alzheimer's disease is under study.

Several proteins are abnormally increased in the brain and appear in the CSF. Whether they are causative or are markers for the disease is not certain. The tau protein (of neurofibrillary origin) has high specificity but low sensitivity for identifying a dementia as Alzheimer's disease; a slightly different type of tau protein also accumulates in patients with progressive supranuclear palsy (see Ch. 179). Choline acetyltransferase is markedly reduced, decreasing the availability of acetylcholine. Somatostatin, corticotropin-releasing factor, and other neurotransmitters are also significantly reduced.

Symptoms and Signs

Alzheimer's disease can be divided into clinical stages. However, patients vary greatly, and disease progression often is not as orderly as the following description implies. The disease progresses gradually, although sometimes symptoms seem to plateau for a time.

The **early stage** is characterized by loss of recent memory, inability to learn and retain new information, language problems (especially word finding), mood swings, and personality changes. Patients may have progressive difficulty performing activities of daily living (eg, balancing their checkbook, finding their way around, or remembering where they put things). Abstract thinking or proper judgment may be diminished. Patients may respond to loss of control and memory with irritability, hostility, and agitation. Some patients have isolated aphasia or visuospatial difficulties. Although the early stage may not compromise sociability, families may report strange behavior (eg, the patient gets lost on the way to the store or forgets the name of a recent dinner guest), accompanied by the onset of emotional lability.

In the **intermediate stage,** patients become unable to learn and recall new information. Memory of remote events is affected but not totally lost. Patients may require assistance with bathing, eating, dressing, or toileting. Behavioral disorganization may be characterized by wandering, agitation, hostility, uncooperativeness, or physical aggressiveness. By this stage, patients have lost all sense of time and place because normal environmental and social cues are used ineffectively. Patients often get lost, sometimes to the point of being unable to find their own bedroom or bathroom. Although they remain ambulatory, they are at risk for falls or accidents secondary to confusion.

In the **severe stage,** patients are unable to walk or to perform any activity of daily living and usually are totally incontinent. Recent and remote memory is completely lost. Patients may be unable to swallow and eat and are at risk of malnutrition, pneumonia (especially from aspiration), and pressure sores. Placement in a long-term care facility often becomes necessary because they are totally dependent on others for care. Eventually, patients become mute. Because such patients cannot relate any symptoms to the physician and because elderly patients often have no febrile or leukocytic response to infection, the physician must rely on experience and acumen whenever a patient looks ill.

Motor or other focal neurologic features occur very late in the disease, although the incidence of seizures is somewhat increased at all stages. The **end stage** of Alzheimer's disease is coma and death, usually from infection.

Complications

Behavioral complications include hostility, agitation, wandering, and uncooperativeness. Psychiatric complications include depression, anxiety, and paranoid reactions. True psychosis (paranoia, delusions, and hallucinations) probably occurs in about 10% of patients with Alzheimer's disease. In addition, perhaps 80% of family members or caregivers develop depression over time. Metabolic problems (eg, dehydration, infection, drug toxicity) can worsen cognitive impairment and make patient management more difficult. Other complications include falls, incontinence, and confusion at dusk (sundowning). The drugs commonly used to treat Alzheimer's disease (especially antipsychotics for behavior disorders) can cause a parkinsonian movement disorder and orthostatic hypotension. Tricyclic drugs with anticholinergic side effects can cause constipation, urinary retention, glaucoma, and seizures. Nonprescription antihistamines can lead to worsened confusion. These complications put the patient at risk of premature institutionalization and should be avoided or quickly treated, because many can be controlled or reversed.

Diagnosis

The diagnosis is usually based on the history, physical examination, laboratory tests, and the exclusion of other causes of dementia. A formal mental status examination should be performed; the Folstein Mini-Mental Status Examination (see FIG. 165–1) is most commonly used. The Barthel scale can

For about 85% of patients with Alzheimer's disease, a correct diagnosis can be made on the basis of a thorough history and results of a standard neurologic physical examination. A brain tissue biopsy is rarely performed or useful.

The essential features of dementia are impairment of short-term memory and long-term memory, abstract thinking, and judgment; other disturbances of higher cortical function; and personality change. Progression of cognitive impairment confirms the diagnosis, and patients with Alzheimer's disease do not improve. The following criteria help establish a probable diagnosis of Alzheimer's disease: dementia established by clinical examination and documented by a formal test of mental status; deficits in two or more areas of cognition; progressive worsening of memory and other cognitive functions; no disturbance of consciousness; onset between ages 40 and 90 yr, most often after age 65; and no systemic or brain disorders that could account for the progressive deficits in memory and cognition. Assessment tools, such as the Hachinski Ischemic Score, can be used to differentiate vascular dementia (see below) from Alzheimer's disease.

The basic evaluation should include a CBC, electrolyte panel measurements, SMA-12/60 (Sequential Multiple Analyzer) tests, thyroid function tests, folate and vitamin B_{12} levels, VDRL test, and urinalysis; ECG and chest x-ray may be useful in some patients. If the history suggests a mass, if focal neurologic signs exist, or if the dementia is of brief duration, CT or MRI should be performed to rule out tumors, infarcts, subdural hematoma, and normal-pressure hydrocephalus. Positron emission tomography is primarily a research technique; however, simple photon emission tomography provides similar information about cerebral perfusion patterns and can contribute to the differential diagnosis in some cases. Lumbar puncture is rarely needed but should be considered if a chronic infection or neurosyphilis is suspected as the cause of cognitive impairment.

Depression, the most common psychiatric problem in the elderly, can closely mimic early-stage Alzheimer's disease and coexists in about 20% of cases; therefore, depression should be considered in patients who present with cognitive impairment.

Prognosis and Treatment

Cognitive decline is inevitable, but the rate of progression is unpredictable. Survival ranges from 2 to 20 yr, with an average of 7 yr.

General treatment principles for Alzheimer's disease are the same as those for all dementias (see Treatment under DEMENTIA, above).

Some drugs that enhance cholinergic neurotransmission, such as donepezil, can at least temporarily improve memory during the early stages of Alzheimer's disease. However, they do not modify the steady worsening of the underlying pathology. Tacrine produces more unwanted side effects. A trial of donepezil starting with 5 mg once daily in the evening and, after 4 to 6 wk, increasing to 10 mg may be considered; it should be continued for several months to assess effectiveness. Antioxidants (eg, vitamin E), estrogen therapy, and NSAIDs are under study.

Many drugs adversely affect the CNS, increasing confusion and lethargy. Sedatives, such as benzodiazepines, should be avoided when possible. Anticholinergic drugs, such as some tricyclic antidepressants, antihistamines, antipsychotics, and benztropine, should be avoided.

An extract of *Ginkgo biloba* called EGb may slow down or modestly reverse memory loss and other symptoms in patients with Alzheimer's disease or vascular dementia. The extract may act as a free-radical scavenger. Complications appear to be minor, but further studies are needed.

NON-ALZHEIMER'S DEMENTIAS

Lewy body dementia may be the second most common dementia after Alzheimer's disease. Lewy bodies are hallmark lesions of degenerating neurons in Parkinson's disease and occur in dementia with or without features of Parkinson's disease. In Lewy body dementia, Lewy bodies may predominate markedly or be intermixed with classic pathologic changes of Alzheimer's disease. Symptoms, signs, and course of Lewy body dementia resemble those of Alzheimer's disease, except hallucinations (mainly visual) are more common and patients appear to have an exquisite sensitivity to antipsychotic-induced extrapyramidal adverse effects.

The next most common [illegible] the elderly is **vascular dementia,** which may coexist with Alzheimer's disease. Other causes are listed in TABLE 171–4.

A patient with non-Alzheimer's dementia sometimes presents similarly to one with Alzheimer's disease. Sometimes, areas of higher function—including speech (aphasia), motor activity (apraxia), interpretation of sensory input (agnosia), judgment, short-term memory, personality, and behavior—are specifically impaired.

In contrast to the cognitive and behavioral changes in Alzheimer's disease, changes in other forms of dementia can occur suddenly and do not necessarily progress. Focal neurologic symptoms (eg, gait abnormalities, seizures), incontinence, and muscle abnormalities may be part of the non-Alzheimer's dementia syndrome and can occur early in its course.

Vascular Dementia

Cerebrovascular disease can destroy enough brain tissue to impair function. Vascular dementia, which includes impairment due to single, strategically located infarcts or to multiple small infarcts from small or medium-sized vessel disease, is more common in men and generally begins after age 70. It occurs more often in persons who have hypertension and/or diabetes mellitus or who abuse tobacco. Progressive vascular dementia can generally be slowed by controlling blood pressure, regulating blood sugar (90 to 150 mg/dL), and stopping smoking. Some degree of vascular damage is found in up to 20% of autopsies of patients with dementia.

Because the pathologic process involves infarction, vascular dementia tends to progress in steps, with each episode accompanied by intellectual decline and often the development of neurologic signs. Cognitive loss may be somewhat focal. In the early stages, personality and insight tend to be better preserved than in Alzheimer's disease. As the disease advances, neurologic signs may develop, especially hemiplegias, pseudobulbar palsy with pathologic laughing and crying, and other signs of extrapyramidal dysfunction.

The symptoms of vascular dementia are sometimes similar to those of Alzheimer's disease, and the two diseases may be difficult to distinguish. An assessment tool, such as [illegible] Early onset (age [illegible] of cigarette smoking, previous strokes, diabetes, heart disease, or hypertension; and the presence of focal neurologic deficits or an intermittent course of clinical progression may help differentiate vascular dementia from Alzheimer's disease. The results of laboratory tests, including CT or MRI, can support but not establish the diagnosis of vascular dementia, but no diagnostic method is foolproof. Even at autopsy, definitive diagnosis is sometimes impossible because the two diseases share some neuropathologic characteristics.

Binswanger's dementia (subcortical arteriosclerotic encephalopathy) is uncommon and involves multiple infarcts in deep hemispheric white matter associated with severe hypertension and systemic vascular disease. Although clinically similar to vascular dementia, Binswanger's dementia may be characterized by more focal neurologic symptoms associated with acute strokes and a more rapid course of deterioration. MRI and CT show areas of leukoencephalopathy in the cerebrum semiovale adjacent to the cortex.

Other Causes

More than 25% of patients with **Parkinson's disease** have dementia; some estimates are as high as 80% (see Ch. 179). At autopsy, patients with Parkinson's disease may have some of the neuropathologic brain findings and many of the biochemical changes seen in patients with Alzheimer's disease. A less severe subcortical dementia is also associated with Parkinson's disease.

The dementia associated with **progressive supranuclear palsy** is commonly preceded by other neurologic symptoms, eg, multiple falls, dystonic axial rigidity, retrocollis, supranuclear ophthalmoplegia, dysphagia, and dysarthria.

Patients with **Huntington's disease (chorea)** may also present with symptoms of dementia, but the diagnosis is usually clarified by the family history, younger age at onset, and the disease's characteristic motor abnormalities (see Ch. 179). In case of doubt, genetic analysis can be diagnostic.

Pick's disease is a less common form of dementia, affecting the frontal and temporal regions of the cortex. Patients have promi-

THE EFFECT OF CELECOXIB, A CYCLOOXYGENASE-2 INHIBITOR, IN FAMILIAL ADENOMATOUS POLYPOSIS

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ABSTRACT

Background Patients with familial adenomatous polyposis have a nearly 100 percent risk of colorectal cancer. In this disease, the chemopreventive effects of nonsteroidal antiinflammatory drugs may be related to their inhibition of cyclooxygenase-2.

Methods We studied the effect of celecoxib, a selective cyclooxygenase-2 inhibitor, on colorectal polyps in patients with familial adenomatous polyposis. In a double-blind, placebo-controlled study, we randomly assigned 77 patients to treatment with celecoxib (100 or 400 mg twice daily) or placebo for six months. Patients underwent endoscopy at the beginning and end of the study. We determined the number and size of polyps from photographs and videotapes; the response to treatment was expressed as the mean percent change from base line.

Results At base line, the mean (±SD) number of polyps in focal areas where polyps were counted was 15.5±13.4 in the 15 patients assigned to placebo, 11.5±8.5 in the 32 patients assigned to 100 mg of celecoxib twice a day, and 12.3±8.2 in the 30 patients assigned to 400 mg of celecoxib twice a day ($P=0.66$ for the comparison among groups). After six months, the patients receiving 400 mg of celecoxib twice a day had a 28.0 percent reduction in the mean number of colorectal polyps ($P=0.003$ for the comparison with placebo) and a 30.7 percent reduction in the polyp burden (the sum of polyp diameters) ($P=0.001$), as compared with reductions of 4.5 and 4.9 percent, respectively, in the placebo group. The improvement in the extent of colorectal polyposis in the group receiving 400 mg twice a day was confirmed by a panel of endoscopists who reviewed the videotapes. The reductions in the group receiving 100 mg of celecoxib twice a day were 11.9 percent ($P=0.33$ for the comparison with placebo) and 14.6 percent ($P=0.09$), respectively. The incidence of adverse events was similar among the groups.

Conclusions In patients with familial adenomatous polyposis, six months of twice-daily treatment with 400 mg of celecoxib, a cyclooxygenase-2 inhibitor, leads to a significant reduction in the number of colorectal polyps. (N Engl J Med 2000;342:1946-52.)

HUMAN colon cancer develops in a stepwise fashion from normal mucosa to adenomatous polyps to carcinoma. Mutation in the adenomatous polyposis coli (*APC*) gene commonly occurs early in the development of sporadic adenomas.[1] Patients with familial adenomatous polyposis have an inherited germ-line *APC* mutation[2] that results in hundreds of adenomatous polyps and a nearly 100 percent risk of colon cancer. Management includes prophylactic proctocolectomy, or colectomy followed by sigmoidoscopic surveillance and rectal polypectomy. Because the adenoma-to-carcinoma sequence in familial adenomatous polyposis resembles sporadic colon carcinogenesis,[1] studies of familial adenomatous polyposis may contribute to the prevention of sporadic adenomas and colon cancer.

Nonsteroidal antiinflammatory drugs (NSAIDs) reduce the incidence of carcinogen-induced colon tumors in rodents.[3,4] NSAIDs are associated with a reduced incidence of and mortality from sporadic adenoma and colon cancer in epidemiologic studies.[5-8] In early clinical studies[9,10] and small, randomized, placebo-controlled trials,[11-13] sulindac caused the regression of colorectal adenomas in patients with familial adenomatous polyposis. However, the gastrointestinal toxicity associated with conventional NSAIDs may limit their long-term use for cancer prevention.[14]

NSAIDs are inhibitors of the cyclooxygenase enzyme family, which catalyzes the metabolism of arachidonic acid to prostaglandins, prostacyclin, and thromboxanes. The cyclooxygenase-1 isoform is constitutively expressed in most tissues, where it medi-

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ates physiologic functions such as gastric mucosal cytoprotection and regulation of platelet aggregation. Its inhibition may account for many of the common side effects of NSAIDs, including gastric ulceration and gastrointestinal hemorrhage.[14,15] The cyclooxygenase-2 isoform is induced in response to cytokines and growth factors and is expressed in inflammatory disease, premalignant lesions (such as colorectal adenomas), and colon cancer.[16-18] Its inhibition has not been associated with gastric ulceration.[15,19-21] However, the long-term effects of selective cyclooxygenase-2 inhibitors as compared with those of traditional NSAIDs remain to be determined.[22] Experimental evidence supports the concept that the chemopreventive effects of NSAIDs may be due at least in part to inhibition of cyclooxygenase-2.[23,24] Hence, selective inhibition of cyclooxygenase-2 offers a potential pharmacologic strategy for the prevention of colorectal adenomas.

To determine whether inhibition of cyclooxygenase-2 could reduce the extent of polyposis in patients with familial adenomatous polyposis, we studied the effect of celecoxib, an agent that selectively inhibits cyclooxygenase-2.[21]

METHODS

Patients

Patients with familial adenomatous polyposis who were 18 to 65 years of age, who had not had their entire colorectum removed, and who had five or more polyps 2 mm or more in diameter that could be assessed endoscopically, were eligible. Exclusion criteria included a history of colectomy within the previous 12 months or colectomy anticipated within 8 months after randomization; use of NSAIDs or aspirin three or more times a week within 6 months of randomization or one or two times a week within 3 months of randomization; or abnormal results of serum laboratory tests (complete blood count and liver-function and renal-function tests).

The study was approved by the institutional review board of the University of Texas M.D. Anderson Cancer Center and the ethics committee of St. Mark's Hospital, London. Written informed consent was obtained from all patients.

Study Design

The study was randomized, double-blinded, and placebo-controlled. It was conducted between December 1996 and December 1998 at the M.D. Anderson Cancer Center in Houston and St. Mark's Hospital in London. One hundred eight patients who were eligible for screening underwent endoscopy; 29 had insufficient polyps for inclusion in the study, and 2 required colectomy for advanced disease (a rectal cancer and a large sessile adenoma). According to the protocol, 75 patients were initially randomly assigned in a 2:2:1 ratio to receive celecoxib (Celebrex, G.D. Searle, Skokie, Ill.), either 100 mg twice daily or 400 mg twice daily, or an identical-appearing placebo orally for six months. The placebo contained 250 mg of lactose. Two additional patients were assigned to the group receiving 100 mg of celecoxib twice daily after two patients were withdrawn because of noncompliance. The study drug and matching placebo were provided by G.D. Searle.

The six-month duration of the study and the end point of adenoma regression were based on previous trials of sulindac that demonstrated an effect on polyp regression within six months of treatment.[9-13] A clinical trial aimed at the prevention of carcinoma, on the other hand, would require many years of study and therefore was not considered feasible for the initial testing of the efficacy of a drug. Evaluations at base line and month 6 included a history taking, physical examination, and endoscopy, with biopsies of the intact or residual colorectum, stomach, and duodenum. Testing for *APC* gene mutations was performed at base line.[25]

Compliance was monitored by means of pill counts and review of diaries completed by the patients. Safety was monitored with a comprehensive symptom questionnaire administered by telephone at two-to-four-week intervals that elicited information on adverse events and by clinical laboratory evaluations at base line and at one, three, and six months. Adverse events were graded in accordance with the National Cancer Institute Common Toxicity Criteria.[26]

Endoscopy

At the base-line endoscopy, an India-ink tattoo was placed in the rectum, colon, or both near a small area with a high density of polyps. The base-line and six-month endoscopic examinations were videotaped, and a series of photographs was taken with the tattoo, appendix, or ileocecal valve positioned centrally and peripherally. These photographs were used for quantitative measurements of the number and size of polyps. Polyps for biopsy were taken from areas that were not photographed for scoring.

Enumeration and Measurement of Polyps

To ascertain that the same area was scored at base line and at month 6, polyps were counted in pairs of photographs. One investigator, other than the endoscopist, who did not know the treatment, performed the scoring. Videotapes were used to resolve ambiguities and confirm polyp counts. The diameter of a polyp was measured with the aid of a standardized endoscopic ruler or biopsy forceps included in the photographic field to serve as a scale. Because in patients with familial adenomatous polyposis the colon is studded with microscopic and poorly visible lesions, only distinct polyps at least 2 mm in diameter were counted.

A qualitative assessment of the total extent of colorectal polyposis was conducted by each of five endoscopists experienced in the management of familial adenomatous polyposis (two from each of the study centers and one from a nonparticipating polyposis center) during joint videotape-review sessions. The first of each pair of videos (obtained at base line and month 6) was scored as the same as, better than, or worse than the second, without the endoscopists' being aware of the temporal sequence or treatment group. A score of "better" or "worse" indicated that there was a clear difference in the total extent of polyp involvement. To avoid bias, videotapes of three colorectal regions (cecum and ascending colon; transverse, descending, and sigmoid colon; and rectum) were assessed separately without the endoscopists' being aware of whether the segments came from the same patient.

Statistical Analysis

All 77 randomly assigned patients were included in the intention-to-treat analysis of toxicity and polyp number, size, and burden. Analysis of the endoscopic videotape assessments was performed in the patients for whom the requisite videotapes were available.

The quantitative response variables were the percent change from base line in polyp number and polyp burden, defined as the sum of the polyp diameters. The percent change in each patient was calculated on the basis of the photographs at the tattoo, appendix, and ileocecal valve, and the mean change was then calculated for each study group. Efficacy was evaluated by comparing the mean percent change from base line in each treatment group with that in the placebo group by the Wilcoxon rank-sum test.

Whether treatment affected the polyp count at six months was also analyzed in a multivariate linear regression model with adjustment for base-line covariates. Two variables indicating the treatment (100 or 400 mg twice a day) were included in the model, and the other base-line covariates were the number of polyps, sex, age, study site, and surgical status (whether the patient had previously

undergone colectomy). We employed a logarithmic transformation of both the base-line and the final polyp-count values to eliminate the skewness in that distribution.

In the qualitative assessment of response, based on review of the endoscopic videotapes, each segment was assigned a score of 1 for better, 0 for same, or −1 for worse, and the mean of the five physicians' scores for each treatment group was compared with that for the placebo group with use of the Wilcoxon rank-sum test. The response of each videotaped colorectal segment (cecum and ascending colon; transverse, descending, and sigmoid colon; and rectum) was analyzed separately. In addition, the response of the total colorectum, defined for each patient as the mean score for all colorectal segments assessed, was analyzed.

Adverse events, including those with an onset within 30 days after the end of treatment, were coded according to World Health Organization Adverse Reaction Terminology and graded for severity with the National Cancer Institute Common Toxicity Criteria.[26] Clinical laboratory data were compared between treatment groups by one-way analysis of variance applied to the change from base line to month 1, month 3, month 6, or early termination.

The Kruskal–Wallis test was used to compare base-line continuous variables among the three treatment groups, and the chi-square test or Fisher's exact test was used to examine associations between nominal variables. All tests were two-sided, and a P value of less than 0.05 was considered to indicate statistical significance.[27] No interim analyses were performed.

RESULTS

Patients

Seventy-seven patients were enrolled: 36 at the M.D. Anderson Cancer Center and 41 at St. Mark's Hospital. The treatment groups were similar with regard to race or ethnic group, sex ratio, surgical status, and number of polyps, but they differed in age: the group assigned to 400 mg of celecoxib twice a day was younger (33.1 years) than the group assigned to 100 mg of celecoxib twice a day (38.6 years) and the placebo group (39.9 years) (Table 1). Sixty-six patients had an identified *APC* mutation, and two additional patients had relatives with known *APC* mutations. Seventy-two of the 77 patients completed the treatment. More than 90 percent of the patients who completed the study took at least 80 percent of the study drug. At base line, the placebo group had a mean (±SD) of 15.5±13.4 polyps, the group assigned to 100 mg of celecoxib twice a day had a mean of 11.5±8.5 polyps, and the group assigned to 400 mg of celecoxib twice a day had a mean of 12.3±8.2 polyps in the focal areas where polyps were counted (P=0.66 for the comparison among groups).

TABLE 1. BASE-LINE CHARACTERISTICS OF THE PATIENTS WITH FAMILIAL ADENOMATOUS POLYPOSIS.*

CHARACTERISTIC	PLACEBO (N=15)	100 mg OF CELECOXIB TWICE DAILY (N=32)	400 mg OF CELECOXIB TWICE DAILY (N=30)	P VALUE
Age — yr	39.9±11.3	38.6±10.0	33.1±10.9	0.04†
Sex — no. (%)				0.84‡
Male	9 (60)	17 (53)	18 (60)	
Female	6 (40)	15 (47)	12 (40)	
Race or ethnic group — no. (%)				0.87§
Black	0	1 (3)	1 (3)	
White	15 (100)	29 (91)	26 (87)	
Hispanic	0	2 (6)	3 (10)	
Height — cm	171.5±7.7	169.9±9.7	169.1±11.6	0.74†
Weight — kg	74.6±16.4	74.4±12.7	71.1±15.4	0.39†
Surgical status — no. (%)				0.45‡
Intact colon	5 (33)	8 (25)	12 (40)	
Colectomy	10 (67)	24 (75)	18 (60)	
No. of polyps	15.5±13.4	11.5±8.5	12.3±8.2	0.66†
Polyp size — mm	2.9±0.5	2.9±0.7	2.9±0.6	0.63†
Polyp burden — mm¶	44.7±36.5	34.8±28.1	37.6±29.4	0.65†

*Plus–minus values are means ±SD.

†The P value was calculated by the Kruskal–Wallis test.

‡The P value was calculated by the chi-square test.

§The P value was calculated by Fisher's exact test.

¶The polyp burden was calculated as the sum of the polyp diameters.

Response to Treatment

Treatment with 400 mg of celecoxib twice daily for six months was associated with a significant reduction from base line in the number of colorectal polyps as compared with the placebo group (28.0 percent vs. 4.5 percent, P=0.003) (Table 2 and Fig. 1). The group receiving 100 mg of celecoxib twice daily had a reduction of 11.9 percent as compared with 4.5 percent in the placebo group (P=0.33). Multivariate linear regression analysis confirmed that 400 mg of celecoxib twice daily reduced the number of colorectal polyps (P=0.005) after adjustment for age, sex, surgical status (colectomy vs. intact colon), number of polyps at base line, and investigational institution.

A reduction of 25 percent or more in the mean number of colorectal polyps was seen in 53 percent of the patients in the group receiving 400 mg of celecoxib twice daily (P=0.003 for the comparison with placebo), 31 percent of the patients in the group receiving 100 mg of celecoxib twice daily (P=0.08), and 7 percent of patients in the placebo group. Intention-to-treat analysis of the specific response of rectal polyps as distinct from colonic polyps showed a mean reduction in the number of rectal polyps of 22.5 percent (P=0.01 for the comparison with the placebo group) in the group receiving 400 mg of celecoxib twice daily and of 3.4 percent (P=0.52 for the comparison with the placebo group) in the group receiving 100 mg of celecoxib twice daily, as compared with a mean increase of 3.1 percent in the placebo group (Table 2).

Whereas the number of polyps was quantified in designated small areas adjacent to a tattoo or anatomical landmark, the full extent of colorectal polyposis was assessed qualitatively from videotapes of complete anatomical segments of the colorectum by a panel of five endoscopists. The videotapes showed that in the group receiving 400 mg of celecoxib twice daily, sig-

TABLE 2. PERCENT CHANGE FROM BASE LINE IN THE MEAN NUMBER OF POLYPS AND COLORECTAL POLYP BURDEN IN PATIENTS WITH FAMILIAL ADENOMATOUS POLYPOSIS TREATED WITH PLACEBO OR CELECOXIB FOR SIX MONTHS.*

VARIABLE	PLACEBO (N=15)	100 mg OF CELECOXIB TWICE DAILY (N=32)	400 mg OF CELECOXIB TWICE DAILY (N=30)
Percent change in no. of colorectal polyps	−4.5±16.4	−11.9±30.3	−28.0±24.0
P value		0.33	0.003
Percent change in colorectal polyp burden†	−4.9±17.3	−14.6±31.7	−30.7±25.7
P value		0.09	0.001
Percent change in no. of rectal polyps‡	+3.1±31.1	−3.4±35.0	−22.5±26.0
P value		0.52	0.01

*Plus–minus values are means ±SD. P values are based on the two-sample Wilcoxon statistic for the comparison of celecoxib with placebo, in the intention-to-treat analysis. Negative numbers indicate decreases, and positive numbers increases.

†The colorectal polyp burden was calculated as the sum of the polyp diameters.

‡Seven subjects had no rectal polyps at base line or on final evaluation. These subjects are considered to have had 0 percent change.

nificant improvement in polyposis occurred in the rectum (P=0.01), in the ascending colon and cecum (P=0.02), and in the transverse, descending, and sigmoid colon (P=0.003) (Table 3). The corresponding changes in the group receiving 100 mg of celecoxib twice daily were not significant, but there was a trend toward a dose response in the rectum (P=0.07) and in the ascending colon and cecum (P=0.10). The combined assessments from all the videotapes of the colon and rectum showed a consistent improvement in the group receiving 400 mg of celecoxib twice daily (P<0.001) as well as in the group receiving 100 mg twice daily (P=0.03).

To estimate changes in polyp area, the polyp burden was calculated as the sum of the polyp diameters. The average decreases in polyp burden were 30.7 percent for the group receiving 400 mg of celecoxib twice daily, 14.6 percent for the group receiving 100 mg of celecoxib twice daily, and 4.9 percent for the placebo group (P=0.001 for the comparison of 400 mg of celecoxib twice daily and placebo) (Table 2).

Safety

Both doses of celecoxib were well tolerated. Sixty-eight percent of the patients in the placebo group, 56 percent of the patients receiving 100 mg of celecoxib twice daily, and 57 percent of the patients receiving 400 mg of celecoxib twice daily reported one or more adverse events of grade 2 or higher according to the National Cancer Institute Common Toxicity Criteria.[26] Of these, the most commonly reported (by at least 10 percent of patients in each treatment group) were diarrhea (placebo, 13 percent; 100 mg of celecoxib twice daily, 19 percent; 400 mg of celecoxib twice daily, 13 percent) and abdominal pain (placebo, 13 percent; 100 mg of celecoxib twice daily, 3 percent; 400 mg of celecoxib twice daily, 7 percent). There were no significant differences in the incidence of any adverse event between the celecoxib groups and the placebo group. In addition to two patients with-

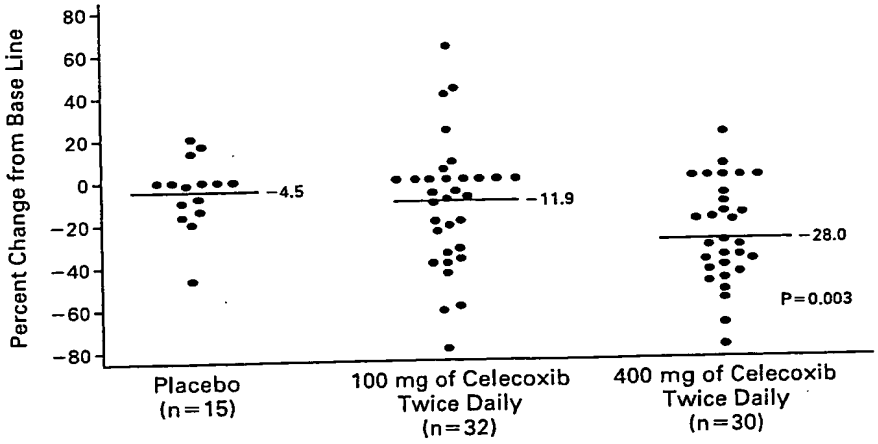

Figure 1. Percent Change from Base Line in the Number of Colorectal Polyps in 77 Patients with Familial Adenomatous Polyposis Who Were Treated with Placebo or Celecoxib (100 mg Twice a Day or 400 mg Twice a Day) for Six Months.

A decrease from base line represents disease regression, and an increase represents disease progression. The horizontal lines show the mean changes. The P value is for the comparison with the placebo group.

TABLE 3. CHANGE IN COLORECTAL POLYPOSIS BASED ON REVIEW OF ENDOSCOPIC VIDEOTAPES IN PATIENTS WITH FAMILIAL ADENOMATOUS POLYPOSIS TREATED WITH PLACEBO OR CELECOXIB FOR SIX MONTHS.*

COLORECTAL SEGMENT	PLACEBO	100 mg OF CELECOXIB TWICE DAILY†	400 mg OF CELECOXIB TWICE DAILY‡
Rectum			
No. of patients	15	29	29
Score	−0.1±0.3	0.2±0.5	0.3±0.4
P value		0.07	0.01
Transverse, descending, and sigmoid colon			
No. of patients	6	3	10
Score	−0.2±0.2	−0.1±0.1	0.4±0.4
P value		0.33	0.003
Cecum and ascending colon			
No. of patients	5	7	10
Score	−0.2±0.4	0.4±0.6	0.5±0.4
P value		0.10	0.02
Total colorectum§			
No. of patients	15	29	29
Score	−0.07±0.26	0.13±0.22	0.33±0.32
P value		0.03	<0.001

*The base-line and six-month evaluations were compared by a panel of endoscopists experienced in the management of familial adenomatous polyposis; these endoscopists assigned scores for anatomical segments at six months as follows: −1 indicated "worse," 0 "no change," and 1 "better." Plus–minus values are means ±SD of the scores for each group. P values are based on the two-sample Wilcoxon statistic for the comparison of celecoxib with placebo, in the analysis of patients for whom the respective videotapes were available.

†Videotapes were not available for three patients.

‡Videotapes were not available for one patient.

§The score for the total colorectum is the mean of the separate assessments of the transverse, descending, and sigmoid colon; the cecum and ascending colon; and the rectum.

drawn for noncompliance, three patients did not complete the study for the following reasons: suicide in a patient in the group receiving 100 mg twice daily with a history of psychiatric disorder and a previous suicide attempt, acute allergic reaction in a patient in the group receiving 400 mg twice daily with a history of allergies, and dyspepsia in a patient in the group receiving 400 mg twice daily. There were no significant alterations in mean laboratory-test values. No ulceration was seen on follow-up esophagogastroduodenoscopy in any patient, including the patient who withdrew because of dyspepsia.

After the study was completed, patients were not offered continuation of treatment with the study drug because the efficacy of the drug was not known until the results were analyzed. Three patients (one from each study group) are known to have undergone colectomy since the completion of the study.

DISCUSSION

In a six-month study, we found that treatment with a cyclooxygenase-2 inhibitor, celecoxib, at a dose of 400 mg twice daily was associated with significant regression of colorectal adenomas in patients with familial adenomatous polyposis. Significant regression was not associated with the dose of 100 mg twice daily. These clinical findings are consistent with other evidence that cyclooxgenase-2 has a role in colonic tumorigenesis and that selective inhibition of cyclooxygenase-2 may help control this process.[23]

Regression of adenomas was seen in the rectum as well as in the left and right sides of the colon. Age and whether or not the patient had undergone colectomy did not affect the results. Nonetheless, our six-month study leaves many important questions unanswered. These include whether prolonged treatment with a medication such as celecoxib can replace, delay the need for, or limit the anatomical extent of proctocolectomy, and whether such treatment can inhibit progression to carcinoma. Our findings suggest, however, that celecoxib could serve as an adjunct to current management by suppressing polyp formation in patients with residual rectum after colectomy and in patients with an intact colon who are awaiting colectomy.

Sulindac, a nonselective cyclooxygenase inhibitor, was previously reported to cause complete or nearly complete regression of rectal adenomas in uncontrolled studies,[9,10,28] and in a small, placebo-controlled, drug-crossover trial of patients with familial adenomatous polyposis.[11] Regression of rectal adenomas, though of lesser magnitude, was reported in two subsequent placebo-controlled studies, by Giardiello et al.[12] and Nugent et al.[13] In the former study, 12 patients treated with sulindac showed maximal improvement by month 6 of the nine-month study. In contrast to earlier reports, no patient had a complete remission, and the clinical effect was considered insufficient to eliminate the need for colectomy in patients with established polyposis. Rapid recurrence of adenomas was also observed three to four months after discontinuation of drug therapy.[11,12] Evidence of long-term efficacy of sulindac is still lacking, and there have been case reports of tumor progression in patients receiving sulindac.[29] Because of differences in patients' characteristics and in study methods, differences in findings among these studies cannot be critically assessed. Long-term studies, as well as direct comparisons of selective and nonselective cyclooxygenase inhibition, could further define the relative clinical benefits of these drugs.

A key question is whether the inhibitory effect of NSAIDs on colon carcinogenesis is mediated by inhibition of either cyclooxygenase-1 or cyclooxygenase-2, or both, or by inhibition of other cellular targets of NSAIDs. Several lines of evidence indicate that cyclooxygenase-2 mediates this process, although noncyclooxygenase pathways may also be involved.[23,30-32] Cyclooxygenase-2 is up-regulated in colonic neoplasms, including adenomas and carcinomas in humans and rodents, and in early adenomas in mice with

germ-line *APC* mutations.[17,24,33] Selective cyclooxygenase-2 inhibition reduces the incidence of carcinogen-induced colonic aberrant crypt foci and carcinomas in rats, as well as the incidence of adenomas in mice with germ-line *APC* mutations.[24,34,35] There is also direct genetic evidence that the cyclooxygenase-2 gene contributed to the development of adenomas in a mouse model of familial adenomatous polyposis, in which knockout of the cyclooxygenase-2 gene greatly reduced the number of intestinal adenomas.[24] Such studies support the concept that the antineoplastic effects of NSAIDs are attributable, at least in part, to inhibition of cyclooxygenase-2.

The specific cellular pathways responsible for the effects of cyclooxygenase-2 on tumorigenesis are under study. Current evidence indicates that cyclooxygenase-2 mediates mitogenic growth factor signaling and down-regulates apoptosis, thus promoting tumor growth.[36-38] The induction of apoptosis by selective inhibition of cyclooxygenase-2 is relevant to familial adenomatous polyposis, in which apoptosis is considered to be attenuated.[39]

Preclinical studies have established the role of cyclooxygenase-2 in colon tumorigenesis and suggested a role for cyclooxygenase-2 inhibition in the prevention of human cancer. Our findings support the application of this strategy to studies of the prevention of colorectal tumors in other populations at risk, including persons with sporadic adenomatous polyps in whom cellular tumorigenesis resembles familial adenomatous polyposis. The role of cyclooxygenase-2 inhibition in preventing adenomas in adolescents with preclinical familial adenomatous polyposis remains to be studied.

Supported by a contract with the National Cancer Institute (NO1 CN-65118), a Cancer Center Support Grant (CA-16672), and contracts with Searle Pharmaceuticals (protocol IQ4-96-02-001).

Drs. Steinbach, Phillips, Wallace, and Levin have served as consultants to G.D. Searle.

We are indebted to Diane Gravel, R.N., for patient care, Ms. Jill Sawyer for genetic counseling, and Nancy Matteer, B.S., for assistance with laboratory tests at the M.D. Anderson Cancer Center; to Kay Neale, Nicola Baxter, and Elizabeth Avery for patient care at the Polyposis Registry of St. Mark's Hospital; to Drs. Randolph H. Bailey, Monica M. Bertagnolli, and Henry T. Lynch for referral of patients; and to the patients and their families for participating in and contributing to this demanding study.

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Clinical Predictive Value of the *in Vitro* Cell Line, Human Xenograft, and Mouse Allograft Preclinical Cancer Models [1]

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ABSTRACT

Purpose: We looked at the value of three preclinical cancer models, the *in vitro* human cell line, the human xenograft, and the murine allograft, to examine whether they are reliable in predicting clinical utility.

Experimental Design: Thirty-one cytotoxic cancer drugs were selected. Literature was searched for drug activity in Phase II trials, human xenograft, and mouse allografts in breast, non-small cell lung, ovary, and colon cancers. Data from the National Cancer Institute Human Tumor Cell Line Screen were used to calculate drug *in vitro* preclinical activity for each cancer type. Phase II activity *versus* preclinical activity scatter plot and correlation analysis was conducted for each model, by tumor type (disease-oriented approach), using one tumor type as a predictor of overall activity in the other three tumor types combined (compound-oriented approach) and for all four tumor types together.

Results: The *in vitro* cell line model was predictive for non-small cell lung cancer under the disease-oriented approach, for breast and ovarian cancers under the compound-oriented approach, and for all four tumor types together. The mouse allograft model was not predictive. The human xenograft model was not predictive for breast or colon cancers, but was predictive for non-small cell lung and ovarian cancers when panels of xenografts were used.

Conclusions: These results suggest that under the right framework and when panels are used, the *in vitro* cell line and human xenograft models may be useful in predicting the Phase II clinical trial performance of cancer drugs. Murine allograft models, as used in this analysis, appear of limited utility.

INTRODUCTION

Both basic science studies and clinical trials are essential components of the cancer drug discovery process. Potential therapeutics found to be significantly better than no treatment or standard therapies (*i.e.*, active) in preclinical laboratory cancer models or compounds with novel chemotypes and equivalent effectiveness to standard treatments are advanced to confirmatory testing in early (Phase I and II) clinical trials. Considering that RR[3] is a reasonable surrogate end point for survival (required but not sufficient), a favorable RR in Phase II trials advances a drug into additional clinical testing and is considered a prerequisite of drug success in the clinic.

Advancing of a candidate drug from preclinical testing in the laboratory to testing in Phase II clinical trials is based on the assumption that drug activity in cancer models translates into at least some efficacy in human patients, *i.e.*, that cancer laboratory models are clinically predictive. In addition, the relevance of tumor type-specific preclinical results for the corresponding human cancers in the clinic can be viewed through two different approaches: compound-oriented, where a drug is assumed to have potential activity against all human tumor types if it is effective against a single test tumor type, and disease-oriented, where a drug with preclinical activity in a single tumor type would only be expected to be effective in the same tumor type in patients.

Although widely adopted, the above-mentioned assumption and approaches have not been confirmed by studies to date. In addition, all studies aimed to examine the clinical predictive value of laboratory cancer models inevitably suffer from inherent bias because compounds with no activity in preclinical models are generally not advanced to clinical trials.

This work was undertaken to examine the clinical predictive value of three preclinical cancer models that have found wide use: the human *in vitro* cell line; the mouse allograft; and the human xenograft. In these models, tumor volume or life span (*in vivo* mouse models) or cell growth (*in vitro* cell lines) is compared between the treatment group receiving the new drug and a control group (active or inactive control).

The use of preclinical cancer models for selection of potential cancer therapeutics was pioneered by the NCI in the United States in the mid-1950s. The screening strategies used until 1990 were essentially compound oriented and involved a

Received 3/26/03; revised 6/ /03; accepted 6/4/03.
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[1] Supported in part by the National Cancer Institute of Canada. Clinical Trials Group. Presented in part at the 2002 Annual Meeting of the American Society of Clinical Oncology (Abstract 360).
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[3] The abbreviations used are: RR, response rate; NCI, National Cancer Institute; NSCLC, non-small cell lung cancer; NSC, National Service Center; T/C%, treated over control tumor volume ratio.

small number of predominantly murine allograft tumors, with emphasis on leukemia (1–7). Several studies from the NCI and others demonstrated that this approach had low clinical predictive value for activity in Phase II trials (5–9) and yielded compounds with selective activity toward human leukemias and lymphomas (10–12). Thus, in 1990, the NCI introduced a disease-oriented *in vitro* Human Tumor Cell Line Screen comprised of 60 cell lines from the most common adult tumors (13–17). The screen was designed so that each tumor type was represented by a panel of cell lines, selected on the basis of different subhistological features, and common drug resistance profiles. It was hoped that this screen would help identify drug leads with high potency and/or selective activity against particular tumor types.

Recently, the NCI examined the correlation between drug activity in Phase II clinical trials and preclinical activity in cancer models (18). Important findings were: (*a*) with the exception of NSCLC, preclinical activity in human xenografts of a particular tumor type did not correlate significantly with Phase II activity in the same type of tumor, (*b*) with the exception of breast and colon histologies, human xenografts did not significantly predict Phase II clinical activity in other cancers types; and (*c*) compounds that were active in at least one-third of all tested human xenografts were likely to have at least some activity in Phase II clinical trials.

Studies examining the clinical predictive value of preclinical cancer models outside the scope of the NCI screening programs have focused on the human xenograft model and have looked predominately into same-tumor correlations (disease-oriented approach). These studies have produced both positive (the model was found clinically predictive) and negative (the model was found to have no clinical predictive value) results in various tumor types (19–27).

Two major criticisms can be made on the overall body of literature concerning the clinical predictive value of preclinical cancer models. First, the vast majority of studies to date, both within and outside the NCI, have based their conclusions on the observation of trends rather than the use of statistical methods. Second, all studies conducted previously have used dichotomous definitions of preclinical and/or clinical activity based on largely invalidated cutoff values of measures of activity: a 20% RR in Phase II clinical trials and (most commonly) a 42% T/C% in human xenografts and mouse allografts.

In addition, two important questions have not been addressed at all by previous studies: the clinical predictive value of the *in vitro* cell line model and the relative clinical usefulness of the different preclinical cancer models in use today (*i.e.*, how different models compare with each other in terms of their ability to identify clinically effective drugs).

Thus, we conducted a study comparing the clinical (Phase II) predictive value of three widely used preclinical laboratory cancer models, the *in vitro* human cell line, the mouse allograft, and the human xenograft. We used quantitative measures of both clinical and preclinical activity and statistical methods. We considered three relevant questions: (*a*) the clinical predictive value of the three models within the same tumor type (disease-oriented approach); (*b*) the clinical predictive value of the three models when one preclinical tumor type is used as a predictor of overall clinical activity in all other tumor types (compound-oriented approach); and (*c*) the clinical predictive value of the three models when overall preclinical and clinical activity in all tumor types combined is considered.

MATERIALS AND METHODS

Study Design

A retrospective, literature-based study was conducted. Data were retrieved from studies published between 1985 and 2000. This period was chosen as one when all three preclinical cancer models of interest to this study were in use and because it was long enough and close enough to the present as to afford data on a relatively large number of recently developed drugs.

The data search was restricted to four of the most common and commonly studied solid tumor types, breast, colorectal, ovarian, and non-small cell lung cancers, to ensure that sufficient data would be available.

The Medline and CancerLit databases were used for the collection of published data. In an attempt to minimize publication bias, both paper publications (peer reviewed) and meeting abstracts (nonpeer reviewed) were used as sources of information. If published data were not available for identified drugs, manufacturers were contacted for unpublished data.

Selection of Drugs

Drugs were identified by searching the Medline and CancerLit databases for compounds that had undergone single agent Phase I clinical trial testing either in 1991 or 1992. Agents with novel targets such as signal transduction or angiogenesis modulators were not included.

This Phase I-based approach to agent identification was used to ensure selection of agents developed within the study time frame of 1985–2000: agents with a published Phase I clinical trial in 1991 or 1992 were expected to have been through preclinical testing between 1985 and 1990 and to have undergone Phase II clinical evaluation by the year 2000. In addition, this approach was adopted to minimize publication bias: publication of Phase I trials is generally less dependent on the observation of favorable tumor responses than publication of Phase II trials or of preclinical cancer model experiments.

Data Collection and Drug Activity

Phase II Clinical Trials. Phase II clinical trials for each drug were identified by searching the Medline and CancerLit databases for scientific papers, reviews, or meeting abstracts. Duplicate publications were discarded. For trials with only abstract information, an additional search by author and/or institution name was conducted in Medline or CancerLit. Scientific papers were used in preference to abstracts, where possible.

Two restrictions were applied. The first was a geographic restriction: to ensure uniform methodology in trial conduct and RR assessment, only Phase II trials conducted in the Americas, Western Europe and Australia were included in the analysis. The second restriction referred to the treatment population and aimed to ensure that uniformly responsive populations of patients would be considered. For breast and ovarian cancer, only Phase II trials that included patients who had received prior chemotherapy for metastatic disease were used, whereas for

NSCLC and colon cancers. the Phase II trials selected included patients who had received no prior chemotherapy.

For each individual Phase II trial the following information was collected: disease site; previous chemotherapy; disease stage; number of patients entered; eligible; evaluable and evaluable for response; number of complete and partial responses; and criteria used for response (standard WHO *versus* other). Trials had to have enrolled a minimum of 14 patients, at least 12 of whom must have been evaluable for response. Completed Phase II trials for which >20% of entered patients were listed as inevaluable for response were considered methodologically unacceptable and were not used. For trials in progress at the time of reporting (meeting abstract format only), the available data were used even if they represented <80% of the enrolled patients, provided that they met the 14-patient criterion. If a trial publication did not specify the previous chemotherapy treatment status of patients, it was not used. Information from Phase I-II trials was used only when the Phase I and II components of the trial were separately conducted and reported. Phase II information was collected regardless of drug dose and route of administration.

For a given drug, in a given cancer type, the activity in a single Phase II clinical trial was recorded as the RR: the number of partial and complete tumor responses over the total number of patients evaluable for response. The number of evaluable rather than eligible patients was used to accommodate information from trials for which final results were not available. In the very few cases where the number of patients evaluable for response was not provided, the number of evaluable patients, the number of eligible patients, or the number of patients entered in the trial (whichever was provided by the investigators) in that priority order was used.

To obtain a drug's overall clinical activity in multiple Phase II trials of patients with the same tumor type, all responses and the collective number of patients evaluable for response were pooled from individual trials to calculate an overall RR. Finally, to get the Phase II activity for any three or four cancer types combined, the individual tumor RRs were averaged.

Human Xenografts and Mouse Allografts. The search strategy for mouse cancer model data were similar to the Phase II process. The only exclusion in this case were results obtained with mouse tumors that were engineered to have special characteristics such as, for example, overexpression of proteins conferring drug resistance.

For each murine allograft or human xenograft, numerical value(s) of activity for drugs of interest was retrieved only if expressed as the treated over control tumor volume ratio (T/C%) or the tumor volume growth inhibition ratio (GI%; and T/C% = 100% − GI%) in the literature sources. In addition, only T/C% values calculated by the formula T/C% = [(RVtreated)/(RVcontrol)] × 100% were collected (where RV = relative volume), whereas T/C% values defined for regressions [T/C% = [(RVtreated (0) − RVtreated(t))/RVtreated (0)] × 100%] were excluded to ensure uniform calculation methods. If the T/C% was not provided but a relative tumor growth curve was given as a figure in a publication, the numerical values for the treatment and control groups provided in this graph were used to calculate the T/C%. Activity reported as all mice cured or 100% complete responses was considered equivalent to and recorded as a T/C% = 0. If no exact T/C% value was given but an interval of values was provided instead (*i.e.*, T/C% >42), a T/C% equal to the interval midpoint value (*i.e.*, a T/C% = 71) was assigned. Finally, where preclinical activity was reported as GI%, it was converted to T/C% by the formula T/C% = 100% − GI%. The activity value for the most effective, nontoxic dose in each schedule was recorded.

Single tumor type preclinical activity of each drug in the murine allograft or human xenograft models was defined as the mean T/C% value from all tested allografts/xenografts of that tumor type. Where the same laboratory had tested a single xenograft/allograft with multiple schedules of the same drug and/or where the same xenograft/allograft had been tested with the same drug by more than one laboratories, T/C% values for a single tumor were obtained by first averaging the same laboratory T/C% values and then the same xenograft T/C% values.

Overall preclinical activity in xenografts/allografts for all four tumor types together was expressed as the average of single tumor mean T/C% values.

***In Vitro* Human Tumor Cell Lines.** The publicly available data from the NCI's Human Tumor Cell Line Screen was used as the information source for the *in vitro* tumor cell line model. Information from the NCI *in vitro* Human Tumor Cell Line Screen was favored because it was a readily available, well-defined, comprehensive, validated, and extensive single source of data. Another important reason was that as an exploratory literature search showed, there was such a wide variation between different investigators in the types of assays used and the nature of cell lines tested that it would have been impossible to comprehensively combine published data from various laboratories.

Acquisition of NCI Human Tumor Cell Line Screen data were done through the internet.[4] Information for each drug was obtained through its NCI code number or NSC number. Such numbers, where available, were identified either from the literature or from a cross-reference of compound names and NSC numbers in the NCI database (also available on the NCI web site).[4]

Testing of compounds in the NCI *in vitro* Human Tumor Cell Line Screen has been described previously (17). Briefly, growth inhibition in cell lines is measured by the GI_{50}, defined as the drug concentration that causes a 50% reduction in cell number in test plates relative to control plates. For every drug entering the screen, a concentration range comprised of five, 10-fold dilutions is tested in each of a group of 60–80 cell lines. The optical densities between treated and control plates, as resulting from the sulforhodamine B assay, are used to construct a dose-response curve for each cell line in the screen, leading to the calculation of a GI_{50} in every case by interpolation. In the case of compounds with low (*i.e.*, the highest concentration tested causes <50% growth inhibition) or high (*i.e.*, the lowest concentration tested causes >50% growth inhibition) potency where interpolation is not possible, the highest and lowest concentrations, respectively, in the tested drug concentration

[4] Internet address: http://www.dtp.nci.nih.gov/docs/cancer/searches/cancer_open_compounds.html.

range are recorded as the approximated GI_{50}s. GI_{50}s are then converted to their Log_{10} values and the overall mean $Log_{10}GI_{50}$ across all cell lines in the screen is calculated. Finally, the results are displayed by a bar graph called the mean graph (28). This graph lists all of the cell lines and their corresponding $Log_{10}GI_{50}$s and relates the magnitude of every individual cell line $Log_{10}GI_{50}$ to the mean $Log_{10}GI_{50}$ across all of the cell lines by a bar to the right (more sensitive than average) or to the left (less sensitive than average) of a vertical line. The experiment is repeated several times for each concentration range. In cases where mean graphs are based on mostly approximated GI_{50}s, other higher or lower concentration ranges of the drug (again made of five, 10-fold dilutions) are also tested. Thus, for each compound tested in the NCI *in vitro* Human Tumor Cell Line Screen, multiple GI_{50} mean graphs (one for each concentration range tested) based on multiple experiments each and with a different content of approximated *versus* calculated (by interpolation) GI_{50}s may exist in the NCI database.

We obtained all of the available GI_{50} mean graph information from the NCI web site for all drugs in our list of compounds with known NSC numbers.[4] For every drug, we recorded the number of concentration ranges tested in the NCI *in vitro* Human Tumor Cell Line Screen, the number of experimental repetitions conducted for each concentration range, and, finally, the number of approximated $Log_{10}GI_{50}$s in each mean graph.

The drug concentration range that produced the mean graph with the smallest number of approximated $Log_{10}GI_{50}$s was used for scoring a drug's activity in the NCI *in vitro* Human Tumor Cell Line Screen, unless a different concentration range existed, with a number of approximated $Log_{10}GI_{50}$s varying <10% from the first but for which more experiments were done.

Preclinical activity in the NCI *in vitro* Human Tumor Cell Line Screen was scored in two different ways: by the mean $Log_{10}GI_{50}$ and by what was termed the activity fraction. For a given drug, in a given tumor type, the mean $Log_{10}GI_{50}$ was computed by averaging the $Log_{10}GI_{50}$s from all of the cell lines of that tumor type in the mean graph corresponding to the most appropriate concentration range. The activity fraction was arbitrarily defined as the number of cell lines of a given tumor type in which the individual $Log_{10}GI_{50}$s were more sensitive to the drug than the average $Log_{10}GI_{50}$ (for all cell lines of all cell types) in the mean graph over the total number of cell lines tested from that tumor type. The activity fraction was also calculated from the mean graph corresponding to the most appropriate concentration range. Overall mean $Log_{10}GI_{50}$s or activity fractions for all four cancer types combined were calculated by averaging the single tumor values.

Statistical Analysis

For each preclinical cancer model, 9 Phase II *versus* preclinical activity relationships were examined for a total of 27 relationships by tumor type (disease-oriented approach, 4 relationships/model), predictive ability of one tumor type for the other three tumor types combined (compound-oriented approach, 4 relationships/model), and general predictive ability for all four tumor types combined (1 relationship/model).

Relationships were first examined descriptively with the construction of various Phase II overall activity *versus* preclinical activity scatter plots (Microsoft Excel software). Each point on these scatter plots represented data from one drug for which both Phase II and preclinical activity values had been calculated from literature sources, as described above.

After descriptive evaluation of the data, Spearman rank correlation coefficients were obtained using the SAS software, UNIX version 6.12. A significance test of every correlation coefficient was performed, and the corresponding *P*s were calculated. Spearman rank (nonparametric) correlation coefficients were used because the distributions of the *x* (preclinical activity) and *y* (clinical activity) variables were not normal (29).

When multiple comparisons are made within a group of data such as in this work, there is increased possibility that some correlations will come up as statistically significant solely because of chance (false positives). To avoid this, multiple comparison correction methods (*e.g.*, Bonferroni approach) are often used to adjust the significance level to a lower *P* than conventionally used. However, relying on corrected probabilities increases the possibility that meaningful correlations will be missed (false negatives), making the nature of the scientific work key to the decision to use multiple comparison adjustment methods or not. Because this was an exploratory study, we were willing to accept a higher probability of false positives to ensure that potentially meaningful associations would not be discarded. We therefore did not correct for multiple comparisons and chose a level of significance of 0.05.

Table 1 Drugs selected for data collection, NSC numbers are shown, where available

Drug	NSC number
Taxotere	628503
Paclitaxel	125973
Topotecan	609699
Irinotecan	
Rhizoxin	332598
Gemcitabine	
Fazarabine	281272
Teniposide	122819
Menogaril	269148
Fosquidone	D611615
Elsamitrucin	369327
Amonafide	308847
Didemnin B	325319
Suramin	
Raltitrexed	639186
Flavone acetic acid	347512
Epirubicin	256942
CI-921	343499
Trimetrexate	352122
Multitargeted antifol	
Vinorelbine	
Piritrexim	351521
Fotemustine	
CI-980	
Chloroquinoxaline sulfonamide	339004
Ilmofosine	
CI-941	
Tiazofurin	286193
Pyrazine diazohydroxide	361456
Tallimustine	
Crisnatol	

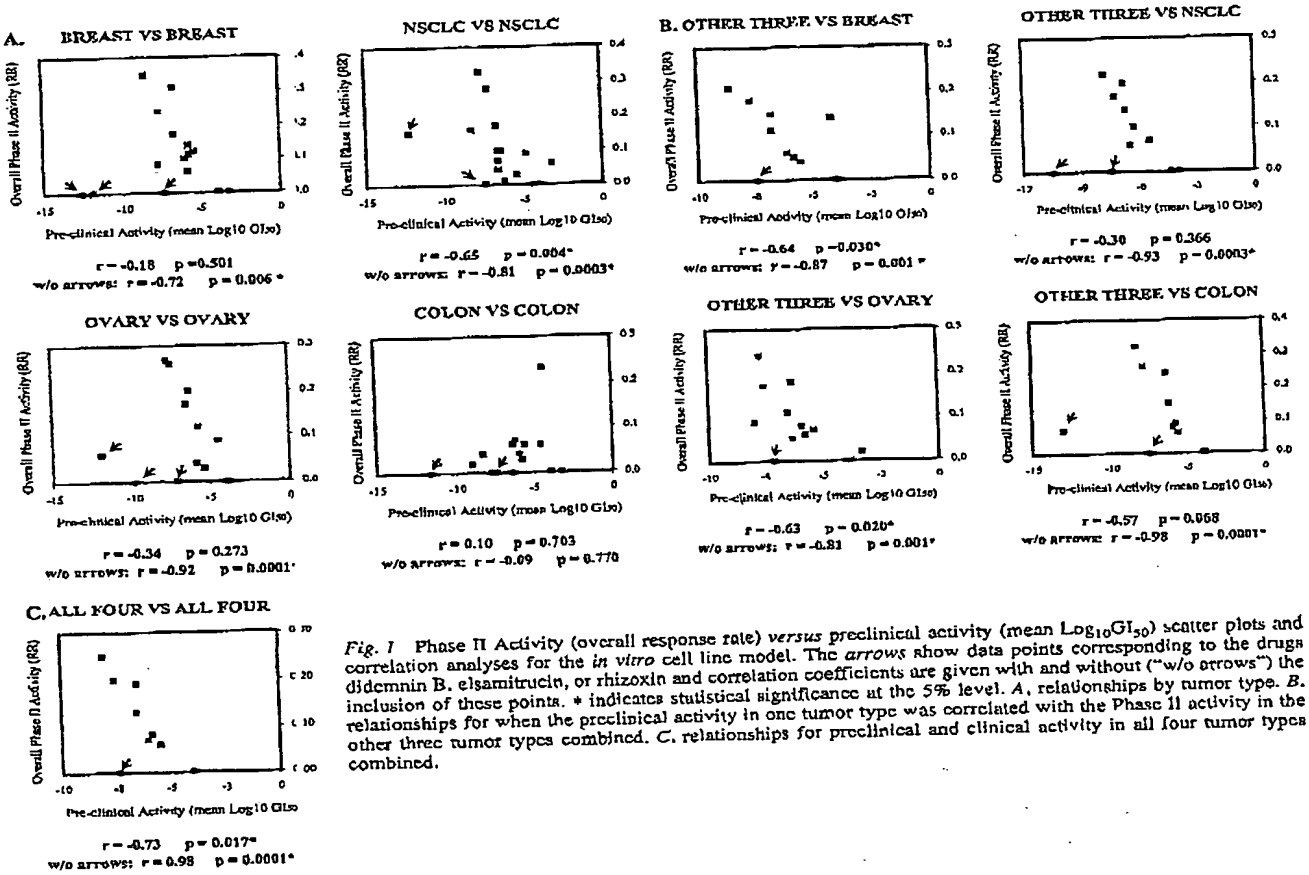


Fig. 1 Phase II Activity (overall response rate) *versus* preclinical activity (mean $Log_{10}GI_{50}$) scatter plots and correlation analyses for the *in vitro* cell line model. The *arrows* show data points corresponding to the drugs didemnin B, elsamitrucin, or rhizoxin and correlation coefficients are given with and without ("w/o arrows") the inclusion of these points. * indicates statistical significance at the 5% level. *A*, relationships by tumor type. *B*, relationships for when the preclinical activity in one tumor type was correlated with the Phase II activity in the other three tumor types combined. *C*, relationships for preclinical and clinical activity in all four tumor types combined.

RESULTS

The Medline and CancerLit databases were searched for cancer drugs (excluding agents with novel targets such as signal transduction or angiogenesis modulators) that had undergone single agent Phase I clinical trial testing either in 1991 or 1992. This search led to 97 drug names. After excluding drugs that were eliminated from additional clinical testing for practical reasons (for example difficulties with the drug formulation), drugs that were specifically developed for a certain type of cancer (as for example hormone-regulating compounds for breast cancer) and drugs that were still the subject of published Phase I studies in 1991 and 1992 despite already being licensed for human use before 1985, a list of 31 agents was obtained (Table 1). After applying the restrictions and criteria mentioned under "Materials and Methods," we extracted from the literature preclinical and Phase II activity information for those agents on four common cancer types, breast, NSCLC, ovary, and colon. Overall, 100 preclinical and 307 Phase II clinical literature references were used spanning the period between 1985 and 2000.

No preclinical data were found for 5 of the 31 drugs researched. Of the 26 drugs remaining, availability of preclinical and Phase II data varied, depending on which preclinical and clinical tumor(s) had been tested and published in each case. Thus, each of the relationships examined had a different number of data points as different subsets of drugs were included. The most data points for any relationship were 17. For six relationships, five or fewer data points were available (relationships with fewer than five data points were not included in the results presented below).

***In Vitro* Cell Line Model.** Fig. 1 shows the Phase II activity *versus* preclinical activity scatter plots and correlation analysis for the *in vitro* cell line model when the mean $Log_{10}GI_{50}$ was used as the measure of preclinical activity. Because the lower the mean $Log_{10}GI_{50}$, the higher the potency of a drug, a negative correlation between mean $Log_{10}GI_{50}$ and Phase II overall RR was expected if the model had a good clinical predictive value. Significant negative correlations were found for NSCLC (Fig. 1*A*), for breast or ovarian cell lines *versus* overall Phase II activity in the other three tumor types

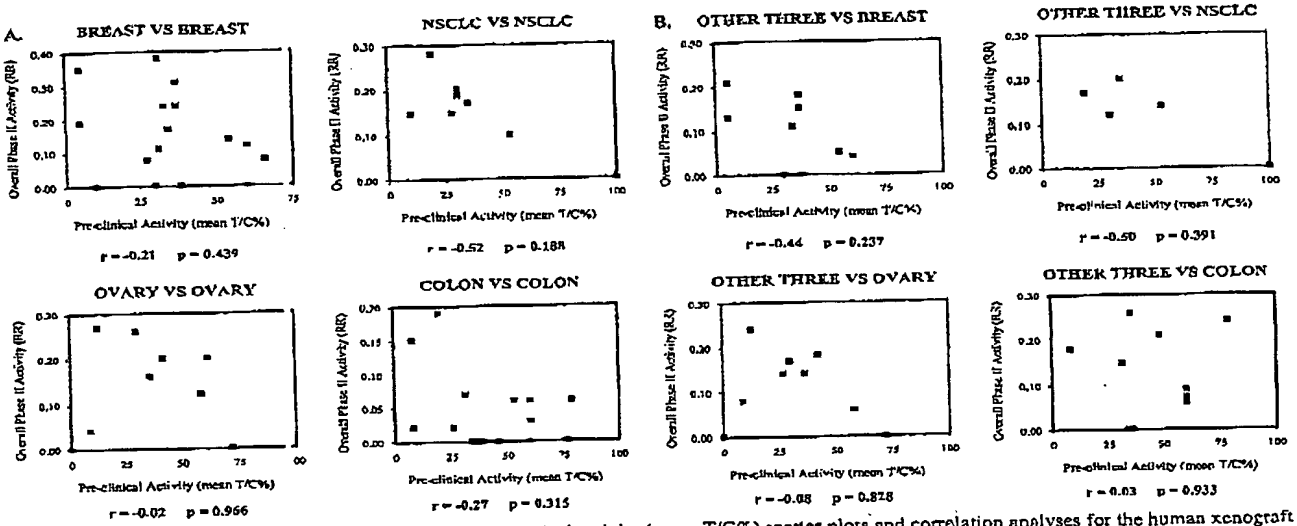


Fig. 2 Phase II activity (overal response rate) *versus* preclinical activity (mean T/C%) scatter plots and correlation analyses for the human xenograft model. *A*, relationships by tumor type. *B*, relationships for when the preclinical activity in one tumor type was correlated with the Phase II activity in the other three tumor types combined.

(Fig. 1*B*), and for preclinical activity *versus* Phase II activity in all four tumor types (Fig. 1*C*).

Although the trends observed with the activity fraction were similar to ones seen for the mean $Log_{10}GI_{50}$ measure, no correlations were statistically significant in this case (data not shown).

Human Xenograft Model. A negative correlation between Phase II RRs and mean T/C% values was expected to be indicative of a good clinical predictive value for the human xenograft model. As shown in Fig. 2, no significant correlations between preclinical and clinical activity were observed for this model in our analysis.

For some of the drugs, preclinical activity calculations were based on multiple human xenografts of the same tumor type (*i.e.*, panels) while for others on only a single xenograft. The relationships in Fig. 2 were reanalyzed, including only the drugs for which preclinical information on more than one human xenograft was available (Fig. 3). The results did not change for breast or colon tumors (compare Fig. 3*A* with Fig. 2*A*). However, the relationship for NSCLC became statistically significant and a highly significant correlation was seen for ovarian cancer (Fig. 3*A*). A near significant correlation was obtained when ovarian human xenograft panels were used to predict clinical activity in the other three tumor types combined (Fig. 3*B*).

Murine Allografts. No significant correlations between preclinical and clinical activity were observed for any of the relationships examined in this study for the murine allograft model (data not shown).

Additional Analyses The scatter plots in Fig. 1 revealed an interesting observation: in every relationship except for colon cancer under the disease oriented approach, an obvious trend toward a negative correlation was evident except for one to three outlier data points (Fig. 1, *arrows*). Interestingly, in all cases, these outlier data points corresponded to the same three drugs, namely elsamitrucin, didemnin B, and rhizoxin.

In an attempt to provide a possible explanation for this observation, we considered the mechanism of action of all drugs that were included in the correlations in Fig. 1. From a total of 18 drugs (Table 2), 5, namely, elsamitrucin, didemnin B, rhizoxin, flavone acetic acid, and fosquidone, were distinct in that they seemed to act through mostly unknown pathways that were not the typical DNA-based mechanisms of action of cytotoxic cancer agents. Thus, although flavone acetic acid and fosquidone fitted the rest of the data, there seemed to be a plausible mechanistic basis for the outlier behavior of the data points for elsamitrucin, didemnin B, and rhizoxin. In fact, exclusion of these three drugs led to highly significant correlations in all cases except for the same tumor relationship in colon cancer (Fig. 1, correlation coefficients and *P*s for "w/o arrows"). It should be noted that none of the relationships examined for the human xenograft models (Figs. 2 and 3) included elsamitrucin, didemnin B, or rhizoxin as data points.

Because of the intriguing results obtained with the human NSCLC and ovarian xenograft panels in Fig. 3*A*, a more detailed examination of these panels was pertained. As seen in Figs. 4*A* and 5*A*, the 6 ovarian and 7 NSCLC xenograft panels differed both in the numbers (minimum of 6 and maximum of 13 for ovary and minimum of 2 and maximum of 8 for NSCLC) and the identity of the xenografts that they contained. Analysis by grade/histology was hindered by lack of complete information on all xenografts. However, some patterns appeared distinguish-

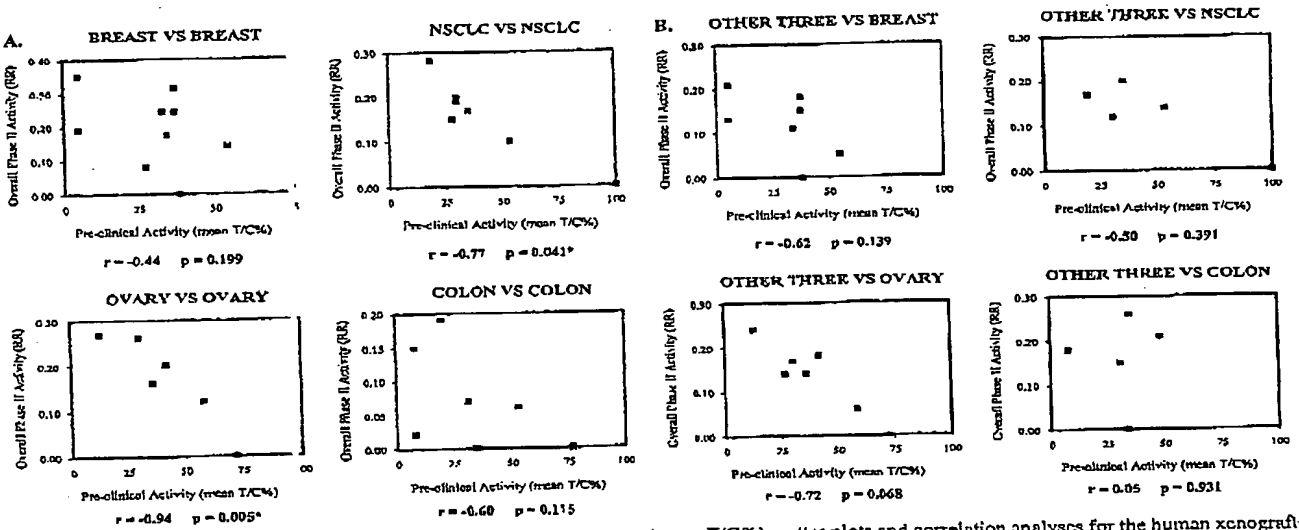


Fig. 3 Phase II activity (overall response rate) *versus* preclinical activity (mean T/C%) scatter plots and correlation analyses for the human xenograft model. Only data points for which two or more human xenografts were used to generate the preclinical activity values are shown. * indicates statistical significance at the 5% level. *A*, relationships by tumor type. *B*, relationships for when the preclinical activity in one tumor type was correlated with the Phase II activity in the other three tumor types combined.

Table 2 Mechanisms of action of drugs used in clinical vs. pre-clinical correlations for the *in vitro* cell line model (Fig. 1)
Atypical cytotoxics are shown in bold.

Drug	Mechanism of action
Amonafide	DNA intercalator
CI-921	Acts on topoisomerase II
Didemnin B	Not understood. Believed to act on protein synthesis
Elsamitrucin	Not understood. It has been observed to inhibit topoisomerase I and II in *in vitro* experiments (relevance to *in vivo* uncertain). In cells in culture it has been observed to cause a cytostatic effect.
Epirubicin	Attaches to DNA at G bases
Fazarabine	Probably inhibits DNA synthesis by incorporation into DNA.
Flavone acetic acid	Has antivascular action in mice (probably not applicable to humans). Also believed to induce cell cycle arrest by generating reactive oxygen species that act on DNA.
Menogaril	Causes cleavage of double-stranded DNA by inhibiting topoisomerase II
Piritrexim	Inhibits dihydrofolate reductase
Rhizoxin	Not fully understood. May interact with tubulin (different binding site than taxoids) and lead to cell cycle arrest. Also observed to act as an angiogenesis inhibitor.
Taxol	Microtubule destabilizing agent that causes apoptosis
Taxotere	Microtubule destabilizing agent that causes apoptosis
Teniposide	DNA synthesis inhibition by stabilization of cleavable DNA complexes
Topotecan	Topoisomerase I inhibitor
Trimetrexate	Antifolate
Fosquidone	Unknown. Not a DNA binder or a topoisomerase inhibitor
Tomudex	Thymidylate synthase inhibitor
Tiazofurin	Inhibits 5'-phosphodehydrogenase, the rate-limiting enzyme for guanine ribonucleotide synthesis

able. All ovarian panels contained 10–20% undifferentiated tumors and also included both poorly differentiated and moderately differentiated subtypes (Fig. 4*B*). For NSCLC, all panels included adenocarcinoma xenografts with a frequency of >30% (Fig. 5*B*). These observations suggested that the frequency of histological/grade subtypes within a xenograft panel may be an important determinant of clinical predictivity rather than the number or the nature of the xenografts.

In an attempt to explore this hypothesis and to further examine the validity of the results obtained for ovarian cancer and NSCLC in Fig. 3*A*, the literature was reviewed for additional data. Six more agents with known overall Phase II RRs in

A.

NAME	HISTOLOGY / GRADE	DATA POINTS (DRUGS)					
		EPIRUBICIN	POSQUIDONE	GEMCITABINE	MENOGARIL	TAXOTERE	PACLITAXEL
MRJ-H-207	undifferentiated	+	+	+	+	+	+
A2780	undifferentiated	+	+	+	+		
Ov.He	mod. diff., mucinous	+	+	+	+		
Ov.Mc	carcinosarcoma	+	+	+	+		
OvRiC	mod. diff., serous	+	+	+	+	+	
Fma	poorly diff., mucinous	+	+	+	+	+	
Ov.Pc	mod. diff., mucinous	+	+	+	+		
Fco	clear cell sarcoma	+	+				
T17	cystoadenocarcinoma	+					
T385	adenocarcinoma	+					
OvGR	mod. diff., mucinous		+			+	
Fko	mod. diff., serous		+	+			
OvG1	poorly diff., serous		+			+	+
OVCAR-3	adenocarcinoma					+	+
A121a	"					+	+
HOC18	poorly diff., serous					+	+
HOC22	poorly diff., serous						+
A2780/DDP	undifferentiated						+
A2780/DX	undifferentiated						+
SKOV-3	adenocarcinoma						+
1° ovary 1	cystoadenocarcinoma						+
1° ovary 2	dediff. serous adenoc.						+
IGROV 1	moderately diff.						+
OVCAR-8	poorly diff. adenoc.						+
OVCAR-5	adenocarcinoma					+	
OvSh	poorly diff., serous					+	
HOC22-S	poorly diff., serous						+
TOTAL NO.		10	10	6	8	10	13

B.

HISTOLOGY/GRADE FREQUENCIES IN HUMAN OVARIAN XENOGRAFT PANELS						
HISTOLOGY / GRADE	EPIRUBICIN NO. (%)	POSQUIDONE NO. (%)	GEMCITABINE NO. (%)	MENOGARIL NO. (%)	TAXOTERE NO. (%)	PACLITAXEL NO. (%)
undifferentiated	2 (20)	1 (10)	1 (17)	2 (25)	1 (10)	3 (23)
mod. diff., mucinous	2 (20)	3 (30)	2 (33)	2 (25)	1 (10)	0 (0)
mod. diff., serous	1 (10)	2 (20)	2 (33)	1 (12.5)	1 (10)	0 (0)
poorly diff., mucinous	1 (10)	1 (10)	0 (0)	1 (12.5)	1 (10)	0 (0)
poorly diff., serous	0 (0)	1 (10)	0 (0)	0 (0)	4 (40)	2 (15)
unspecified	4 (40)	2 (20)	1 (17)	2 (25)	2 (20)	8 (62)
TOTAL	10 (100)	10 (100)	6 (100)	8 (100)	10 (100)	13 (100)

Fig. 4 Human ovarian xenograft panels for the six data points (drugs) used in the "Ovary *versus* Ovary" relationship in Fig. 3*A*. *A*, names and histology/grade (? = unknown, mod. diff. = moderately differentiated, poorly diff. = poorly differentiated, dediff. = dedifferentiated, adnocr = adenocarcinoma) of all of the xenografts tested. Inclusion of a particular xenograft in one of the panels is shown by a "+" sign in the corresponding row and under the appropriate drug column. *B*, histology/grade subtypes in the human ovarian xenograft panels by number and percentage.

previously treated patients with ovarian cancer were found. Five and one of these compounds had been tested in a panel of 15 and 6 human ovarian xenografts, respectively (26, 30), which fitted the histology/grade patterns identified in Fig. 4*B*. Fig. 6*A* lists the names and Phase II RRs (31–56) of these additional drugs together with the six compounds that were included in the analysis in Fig. 3*A*. Fig. 6, *A* and *B*, also shows mean T/C% values scatter plots and statistical analyses for two cases: first, for when all of the available xenograft information was used, and second, for when mean T/C% calculations were based, where possible, on the arithmetically smallest panel, namely the one used for gemcitabine in Fig. 4. Highly significant correlations were obtained in both cases (Fig. 6*B*).

For NSCLC information on two additional agents was found: amsacrine [mean T/C% of 62 (26) and Phase II RR equal to 0.06 (31)] and doxorubicin [mean T/C% of 47 (26) and Phase II RR equal to 0.12 (32)]. Both had been tested in NSCLC human xenograft panels that included all three histological subtypes and had adenocarcinoma contents of 29 and 33%, respectively. As for ovarian cancer, those two additional data points (Fig. 5*C*, *arrows*) enhanced the statistical significance of the relationship observed in Fig. 3*A*.

DISCUSSION

A literature-based, retrospective study was conducted to examine the clinical predictive value of three widely used preclinical cancer models, namely, the *in vitro* human tumor cell line, the human xenograft, and the murine allograft models. Four solid tumor types were selected, breast, NSCLC, ovary and colon, and data on a set of 31 anticancer agents (excluding agents with novel targets such as signal transduction or angiogenesis modulators) were collected. Preclinical activity in each model was correlated with RRs in Phase II clinical trials by tumor type (disease-oriented approach) in the case when one preclinical tumor type was used as a predictor of overall clinical activity in the other three tumor types combined (compound-oriented approach) and for all four tumor types together.

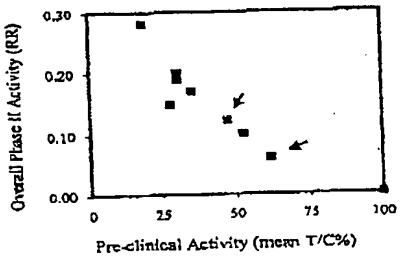
A.

XEN. NAME	XENOGRAFT HISTOLOGY	DRUGS						
		EPI	FAZ	GEM	IRINO	PACLIT	TOPO	VINRLB
T222	squamous cell	+						
T291	adenocarcinoma	+						
UCLA-P3	adenocarcinoma		+					
ACCOLU-78	squamous cell		+					
NCI-H460	large cell			+	+	+	+	+
A549	adenocarcinoma			+	+	+	+	+
CaLu-6	adenocarcinoma			+				
H-74	?			+				
LC-376	?			+				+
QG-56	squamous cell				+			
NCI-H23	adenocarcinoma				+	+	+	
NCI-H226	squamous cell				+	+		
MV-522	adenocarcinoma					+		
CaLu-3	adenocarcinoma					+		
1° NSCLC	adenocarcinoma					+		
L2987	adenocarcinoma					+		+
L-27	adenocarcinoma							+
LC-06	large cell							+
LU-65	large cell							+
PC-12	adenocarcinoma							+
LU-99	large cell							
TOTAL NO.		2	2	5	5	8	3	8

B.

HISTOLOGY FREQUENCY IN HUMAN NSCLC XENOGRAFT PANELS							
HISTOLOGY	EPI NO. (%)	FAZ NO. (%)	GEM NO. (%)	IRINO NO. (%)	PACLIT NO. (%)	TOPO NO. (%)	VINORLB NO. (%)
adenocarcinoma	1 (50)	1 (50)	2 (40)	2 (40)	6 (75)	1 (33.3)	3 (37.5)
large cell	0 (0)	0 (0)	1 (20)	1 (20)	1 (12.5)	1 (33.3)	4 (50)
squamous cell	1 (50)	1 (50)	0 (0)	2 (40)	1 (12.5)	1 (33.3)	1 (12.5)
unknown			2 (40)				
TOTAL	2 (100)	2 (100)	5 (100)	5 (100)	8 (100)	3 (100)	8 (100)

C. NSCLC VS NSCLC (ADDITIONAL DATA)



r = -0.90 p = 0.001*

Fig. 5 Human NSCLC xenograft panels for the seven data points (drugs) used in the NSCLC *versus* NSCLC relationship in Fig. 3*A*. *A*, drug names (EPI = epirubicin, FAZ = fazarabine, GEM = gemcitabine, IRINO = irinotecan, PACLIT = paclitaxel, TOPO = topotecan, VINRLB = vinorelbine) and histological subtypes (? = unknown) of all of the xenografts tested. Inclusion of a particular xenograft in one of the panels is shown by a "+" sign in the corresponding row and under the appropriate drug column. *B*, histological subtypes in the human NSCLC xenograft panels by number and percentage. *C*, scatter plot and correlation analysis for the same tumor clinical *versus* preclinical activity relationship in NSCLC, including the seven drugs in Fig. 6*A* as well as two additional agents, doxorubicin and amsacrine (data points shown with *arrows*), with known NSCLC Phase II and human xenograft activities

Colon cancer was the only site for which a disproportional amount of clinically active *versus* inactive agents were identified: only 3 drugs with Phase II RRs > 0.15 and 8 with ≤0.10 (Figs. 1–3). However, this was likely a reflection of the lack of clinically effective drugs for this tumor type rather than the result of selection and publication bias.

When the mean Log $_{10}GI_{50}$ measure of preclinical activity was used, the *in vitro* cell line model was found to be predictive of Phase II clinical performance for NSCLC under the disease-oriented approach in breast and ovarian cancers under the compound-oriented approach and in the case of all four tumor types together. Highly significant correlations were observed in all cases, except colon cancer, when three consistent outlier data points corresponding to the mechanistically nontypical cytotoxic agents didemnin B, elsamitrucin, and rhizoxin were excluded in exploratory analysis. Thus, the *in vitro* cell line model

A.

	DRUG	PHASE II RESPONSE RATE	HUMAN OVARIAN XENOGRAFT MEAN T/C%	
			ALL TESTED	GEMCITABINE PANEL
STUDY DRUGS	EPIRUBICIN	0.20	42	-
	FOSQUIDONE	0.00	72	-
	GEMCITABINE	0.16	36	36
	MENOGARIL	0.12	58	-
	PACLITAXEL	0.26	30	-
	TAXOTERE	0.27	13	-
ADDITIONAL DRUGS	DOXORUBICIN	0.19 [346]	47 [a]	47 [a]
	AMSACRINE	0.05 [a]	75 [a]	-
	CISPLATIN	0.25 [7,8]	41 [a]	46 [a]
	HEXAMETHYL-MELAMINE	0.19 [9,44]	28 [a]	31 [a]
	METHOTREXATE	0.09 [9,45]	76 [a]	84 [a]
	5-FU	0.10 [9,46]	71 [a]	71 [a]

B.

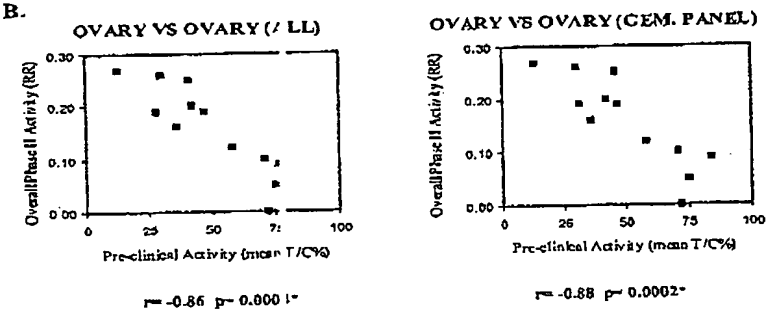


Fig. 6 *A*, preclinical and Phase II clinical activity data for ovarian cancer, including the six drugs in Fig. 3*A* ("Study Drugs") as well as an additional six drugs ("Additional Drugs") with known ovarian Phase II and human xenograft activities. Literature references are shown in superscript font. *B*, scatter plots and correlation analysis for the same tumor clinical *versus* preclinical activity relationship in ovarian cancer based on the data in Fig. 5*A*. Analysis was done for (*a*) when all of the xenografts were included in preclinical activity calculations ("All") and (*b*) when only the six xenografts in the gemcitabine panel were used for preclinical activity calculations, where possible ("Gem. Panel"). Stars indicate statistical significance at the 5% level.

might be predictive in the case of typical cytotoxic cancer agents but might fail to provide reliable information for at least some of the noncytotoxic cancer drugs. Additional studies are needed to explore this observation.

The fact that drug potency (mean $Log_{10}GI_{50}$), a pharmacological measure, was found to be predictive of Phase II performance was somewhat surprising but has been noted previously; a recent study by Johnson *et al.* (18) demonstrated a highly significant correlation between potency in the NCI human tumor cell line screen and activity in the hollow fiber assay. Pharmacological considerations (pharmacological differences between the species) might provide a possible explanation why some anticancer agents appear effective in *in vivo* mouse models but fail to show efficacy in Phase II trials. Experience with some agents (57) has shown that the maximum-tolerated dose in mouse can be higher than in humans, presumably because of an intrinsic ability of mouse cells to tolerate higher drug doses and/or more efficient elimination in the mouse.

In contrast to the *in vitro* cell line, our results suggest that the murine allograft model, as used in this analysis, is not predictive of clinical Phase II performance. This is in agreement with the conclusions from a large body of information originating from the NCI screening programs in use from 1975 to 1990 (5–8, 10–12).

The human xenograft model showed good tumor-specific predictive value for NSCLC and ovarian cancers when panels of xenografts were used. However, it failed to adequately predict clinical performance both in the disease and compound-oriented settings for breast and colon tumors. The results with breast cancer were in agreement with a recent study (18) but were contradictory to the work reported by Bailey *et al.* (20). Inoue *et al.* (21), and Mattern *et al.* (24). However, given that the latter studies did not use formal statistical methods, our conclusions may be more robust. The results for ovarian cancer were in agreement with studies by Tactle *et al.* (23) and Mattern *et al.* (24) but contradicted the conclusions of the recent NCI United States study by Johnson *et al.* (18). Our results for NSCLC were consistent with the observations from all previous studies that examined same tumor correlations in this cancer type (18, 24).

For NSCLC and ovarian cancer patients, a panel of xenografts was more predictive than single xenografts confirming preliminary observations by Bellet *et al.* (19).

In an effort to identify the properties that may render an ovarian or NSCLC human xenograft panel predictive of Phase II drug performance, common characteristics were sought. There was no similarity in number and only limited overlap in identity of xenografts between same tumor type panels. However, certain patterns in histology/grade content were found. These ob-

servations suggest that the relative histology/grade content rather than the number or identity of xenografts within a panel may be the important determinant of clinical predictivity. To our knowledge, no other study has attempted to identify ovarian or NSCLC human xenograft panel features that might lead to accurate predictions of a drug's Phase II performance.

This is the only study that has examined the clinical predictive value of three preclinical cancer models together and thus allows for direct comparisons between them. The results suggest that the human xenograft model is more predictive than its murine allograft counterpart and that the *in vitro* cell line model is of, at least, equivalent usefulness to the human xenograft model.

The NCI work with cancer drug screening programs from 1955 to 1990 (Refs. 5–8, 10–12; leukemia-based preclinical, compound-oriented screens preferentially yielding compounds active against hematological malignancies) in combination with our work and recent conclusions by Johnson *et al.* (Ref. 18; statistically significant results under the compound-oriented approach for some solid tumors) suggest that the compound-oriented strategy may be successful when used only within solid tumors or only within hematological malignancies but not when the two disease groups are considered together.

In general, our results suggest that the *in vitro* human tumor cell line and the human xenograft models might have good clinical predictive value in some solid tumors (such as ovary and NSCLC) under both the disease and compound-oriented strategies, as long as an appropriate panel of tumors is used in preclinical testing.

In conclusion, given the results in this study and those of others (6, 7, 10–12), continued use of the murine allograft model in drug development may not be justified. The work presented here argues for emphasis to be placed on *in vitro* cell lines (in the context of the NCI Human Tumor Cell Line Screen) and appropriate panels of the human xenograft model.

Recent years have seen an explosion in the molecular understanding of cancer, which has led to the development of not only more effective cytotoxic cancer drugs but of potentially cytostatic or antimetastatic agents as well. The future preclinical and clinical development of traditional cytotoxic compounds will likely follow similar procedures with those practiced today, and in that sense, the present findings could contribute to the more efficient discovery of such agents. However, the existing cancer models and parameters of activity in both the preclinical and clinical settings may have to be redesigned to fit the mode of action of the novel cytostatic, antimetastatic, antiangiogenesis, or immune response-modulating agents (58). In the preclinical cancer model front, the case is being made for the use of the orthotopic mouse xenograft and transgenic models (59–61) because those are thought to more accurately simulate human disease, especially in terms of growth characteristics and metastatic behavior. New end points of preclinical activity are contemplated such as the demonstration that a new molecule truly hits the intended molecular target (58). In Phase II clinical trials, there is a growing effort toward validating new surrogate endpoints of drug efficacy (58). The next decade will probably answer many of the questions regarding the effectiveness of these novel agents and will likely define a new role for traditional cytotoxic therapies, but it will also bring new challenges in terms of preclinical predictors of activity.

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EDITORIALS

Aromatase Inhibitors — A Triumph of Translational Oncology

Sandra M. Swain, M.D.

Great strides have been made in the diagnosis and treatment of early-stage breast cancer, thanks to advances in molecular medicine, interdisciplinary treatment, and rapid electronic communication. Hormonal therapy, the first and most successful targeted therapy for breast cancer, has saved many thousands of lives. Moreover, screening and adjuvant (postoperative) therapy have increased survival among women with breast cancer.[1,2] The improvement in survival can be attributed to both adjuvant tamoxifen therapy and adjuvant chemotherapy and has been found in all subgroups of patients regardless of the presence or absence of tumor cells in draining lymph nodes, including women who are premenopausal, those who are postmenopausal, those with estrogen-receptor–negative tumors, and those with estrogen-receptor–positive tumors. Experts are now in the process of classifying breast cancer, which actually consists of a heterogeneous group of cancers, into multiple categories. It is essential to define each subgroup precisely and to delineate distinct characteristics and targets that will lead to tailored therapies that are better than the ones we have now.

In this issue of the *Journal*, the Breast International Group (BIG) 1-98 Collaborative Group reports on a randomized comparison of letrozole, an aromatase inhibitor, with tamoxifen as adjuvant therapy for postmenopausal women with early-stage breast cancer. Their findings validate the results of previous studies showing that aromatase inhibitors were more efficacious than tamoxifen in such women.[3] The BIG 1-98 Collaborative Group found a reduction in the incidence of relapse of 3.4 percentage points at five years in the letrozole group, as compared with the tamoxifen group, after a median follow-up of 25.8 months. The incidence of both distant recurrence and contralateral breast cancers was reduced. The benefit was greatest in patients who had also received chemotherapy, who did not receive radiotherapy, and who had positive nodes. Longer follow-up is important to define the benefit of letrozole in patients with node-negative disease. There was no significant difference in survival between the two groups, but at this point, fewer deaths have occurred among women assigned to letrozole.

Five other large trials have also evaluated aromatase inhibitors. The Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial, with a median follow-up of 68 months, found that, as compared with tamoxifen, adjuvant treatment with anastrozole reduced the recurrence rate by 3.7 percentage points in patients with hormone-receptor–positive tumors.[4] The MA.17 trial, in which women first received tamoxifen for five years and then were randomly assigned to receive placebo or letrozole, found that letrozole improved disease-free survival by 4.6 percentage points, after a median follow-up of 30 months, with a survival difference in the node-positive group only.[5] The Intergroup Exemestane Study (IES), with a median follow-up of 30.6 months, compared 2 to 3 years of tamoxifen followed by 2 to 3 years of exemestane with 5 years of tamoxifen therapy and found that the former regimen increased disease-free survival by 4.7 percentage points.[6] The Italian Anastrozole Trial (ITA), with a median follow-up of 36 months, compared 2 to 3 years of tamoxifen followed by 2 to 3 years of anastrozole with 5 years of tamoxifen and found that sequential treatment re-

duced recurrent-free survival by 5.8 percentage points.[7] Finally, a combined analysis of data from two prospective, multicenter, randomized trials (the Austrian Breast and Colorectal Cancer Study Group trial 8 plus the Arimidex–Nolvadex study) compared women who received two years of tamoxifen followed by three years of anastrozole with women who were given tamoxifen for five years. After a median follow-up of 28 months, sequential therapy was associated with an event-free survival rate that was 3.1 percentage points higher than the rate associated with tamoxifen alone.[8] These five studies varied with respect to the number of women with hormone-receptor–positive tumors, node-negative tumors, and node-positive tumors and the definition of outcomes. It is clear, however, that these trials, with close to 30,000 participants, consistently demonstrate that treatment with an aromatase inhibitor alone or after tamoxifen treatment is beneficial. The questions that remain are the optimal duration of treatment with an aromatase inhibitor, whether tamoxifen or an aromatase inhibitor should be given first, whether sequential treatment is optimal, which aromatase inhibitor is best, and whether an aromatase inhibitor is beneficial for premenopausal women after ovarian ablation. The decrease in contralateral cancers among women treated with an aromatase inhibitor has important implications for chemoprevention. Ongoing trials should answer each of these questions.

One of the most exciting aspects of the findings of these evaluations of aromatase inhibitors is that an animal model predicted the results. In tumor cells and peripheral tissues in postmenopausal women, estrogen is synthesized by aromatase from androstenedione and testosterone. A mouse model was developed to simulate the hormonal milieu in postmenopausal women and used to investigate the ability of aromatase inhibitors and tamoxifen to hinder the growth of breast-cancer cells.[9] This model predicted a superior clinical outcome with aromatase inhibitors. The same model also predicts that the administration of letrozole alone will be more effective than the sequential administration of tamoxifen and letrozole.[10] Future analyses of the continued follow-up of the BIG 1-98 study, which includes a group randomly assigned to receive letrozole before tamoxifen therapy and a group assigned to receive letrozole after tamoxifen therapy, will answer this important question.

A hypothesis developed from the ATAC study is that estrogen-receptor–positive, progesterone-receptor–negative tumors are more susceptible to anastrozole than tumors that have both types of hormone receptors.[11] Although this hypothesis was not supported by the findings of the BIG 1-98 study, because of the relatively short follow-up and multiple subgroup analyses in the study, the idea also cannot be ruled out. Data that support a differential benefit in patients with progesterone-receptor–negative tumors include the finding that patients with such tumors are likely to have HER-1–positive or HER-2–positive breast cancer, positive nodes, tumors with high rates of proliferation and aneuploidy, and lower median levels of estrogen receptors. All these features are typical of an aggressive tumor.[12] Another area of fertile research is the crosstalk between growth factor signaling pathways and the estrogen receptor. This crosstalk may result in tamoxifen resistance by potentiating agonist properties of tamoxifen.

It is clear that unlike tamoxifen, aromatase inhibitors are not associated with an increased risk of thromboembolism or uterine cancer. The incidence of fractures and arthralgias is, however, increased among women taking these inhibitors. Both complications are the result of estrogen deficiency, and they require a thorough evaluation with the aim of limiting these adverse effects. In the BIG 1-98 study, the incidence of serious cardiac events was significantly higher among women given letrozole than among those given tamoxifen. An increase in cardiovascular events among patients receiving an aromatase inhibitor has also been suggested in the IES and ATAC studies. This finding may be due to a cardioprotective effect of tamoxifen, but whatever the mechanism, the potential for adverse cardiovascular events needs close and careful evaluation.

We have seen a substantial increase in the number of patients with small, node-negative tumors over the past several years. In the future, molecular characterization of individual tumors will assist in determining the metastatic potential of the tumor and its sensitivity to various agents. It is our responsibility as physicians to determine the appropriate adjuvant treatment for patients,

but the choices are increasingly complex. Fortunately, we have the results of large, prospective, well-designed, and well-executed clinical trials, such as BIG 1-98, to facilitate our recommendations. We await longer follow-up from all the studies to enable us to offer patients sound advice regarding the benefits and long-term risks of aromatase inhibitors. Meanwhile, all the evidence points to aromatase inhibitors as critically important for improving the outcome among postmenopausal women with breast cancer who have positive or negative lymph nodes and who are at a substantial risk for recurrent disease.

No potential conflict of interest relevant to this article was reported.

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Trial Registration Report Card

Jeffrey M. Drazen, M.D., and Alastair J.J. Wood, M.D.

One measure of medical progress is new treatments. The discovery of a novel therapy takes time and money, but more important, it requires the mutual effort of groups that, while they share the common goal of improved treatment, often have fundamentally competing interests. These interests intersect at the clinical trial. Patients who are looking for more effective and safer treatment agree to take part in a clinical trial in the hope that they will benefit from such treatment or that others with similar conditions will benefit later. The company developing the new therapy shares the hope that the trial will be successful, because it wants to market the tested therapy exclusively and profitably for as long as possible before its competitors can launch a similar therapy into the marketplace. These goals, though overlapping, are inevitably in conflict and will generate tension. Such tension has been thrown into sharp relief over the past 15 months by the push for clinical trial registration.

The academic establishment and patients have argued that when patients, motivated by altruism, participate (or even consider participating) in a clinical trial, they are entitled to understand fully all the options available to them in the various trials that are currently recruiting subjects. In addition, their participation in a clinical trial should result in generalizable knowledge that will be available to future patients and investigators to improve patient care. This can happen only when appropriate details of the clinical trial are made available to the public in a timely fashion. The Internet and public registries have made this possible.

Some in industry have argued that to open

CORRECTION

Aromatase Inhibitors — A Triumph of Translational Oncology

Aromatase Inhibitors — A Triumph of Translational Oncology . On page 2807, the sentence that begins five lines from the bottom of the right-hand column should have read, "The Italian Anastrozole Trial (ITA), with a median follow-up of 36 months, compared 2 to 3 years of tamoxifen followed by 2 to 3 years of anastrozole with 5 years of tamoxifen and found that sequential treatment increased recurrent-free survival by 5.8 percentage points," not "reduced recurrent-free survival," as printed.

N Engl J Med 2006;355:533-a

Arteriosclerosis, Thrombosis, and Vascular Biology

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Troglitazone Inhibits Formation of Early Atherosclerotic Lesions in Diabetic and Nondiabetic Low Density Lipoprotein Receptor–Deficient Mice

Alan R. Collins, Woerner P. Meehan, Ulrich Kintscher, Simon Jackson, Shu Wakino, Grace Noh, Wulf Palinski, Willa A. Hsueh and Ronald E. Law

Arterioscler. Thromb. Vasc. Biol. 2001;21;365-371

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514

 Print ISSN: 1079-5642. Online ISSN: 1524-4636

Troglitazone Inhibits Formation of Early Atherosclerotic Lesions in Diabetic and Nondiabetic Low Density Lipoprotein Receptor–Deficient Mice

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Abstract—Peroxisome proliferator–activated receptor-γ (PPARγ) is a ligand-activated nuclear receptor expressed in all of the major cell types found in atherosclerotic lesions: monocytes/macrophages, endothelial cells, and smooth muscle cells. In vitro, PPARγ ligands inhibit cell proliferation and migration, 2 processes critical for vascular lesion formation. In contrast to these putative antiatherogenic activities, PPARγ has been shown in vitro to upregulate the CD36 scavenger receptor, which could promote foam cell formation. Thus, it is unclear what impact PPARγ activation will have on the development and progression of atherosclerosis. This issue is important because thiazolidinediones, which are ligands for PPARγ, have recently been approved for the treatment of type 2 diabetes, a state of accelerated atherosclerosis. We report herein that the PPARγ ligand, troglitazone, inhibited lesion formation in male low density lipoprotein receptor–deficient mice fed either a high-fat diet, which also induces type 2 diabetes, or a high-fructose diet. Troglitazone decreased the accumulation of macrophages in intimal xanthomas, consistent with our in vitro observation that troglitazone and another thiazolidinedione, rosiglitazone, inhibited monocyte chemoattractant protein-1–directed transendothelial migration of monocytes. Although troglitazone had some beneficial effects on metabolic risk factors (in particular, a reduction of insulin levels in the diabetic model), none of the systemic cardiovascular risk factors was consistently improved in either model. These observations suggest that the inhibition of early atherosclerotic lesion formation by troglitazone may result, at least in part, from direct effects of PPARγ activation in the artery wall. (*Arterioscler Thromb Vasc Biol.* 2001;21:365-371.)

Key Words:: atherosclerosis ■ diabetes mellitus ■ pharmacology

Peroxisome proliferator–activated receptor-γ (PPARγ), a nuclear receptor, is expressed in all major cell types participating in vascular injury: endothelial cells (ECs), macrophages, and vascular smooth muscle cells (VSMCs).[1–6] Activation of this receptor in vitro inhibits inflammatory processes, including cytokine production and expression of NO synthase.[2] In early clinical investigations, ligands of PPARγ, such as thiazolidinediones (TZDs), have also been reported to improve endothelium-dependent vasodilation, suggesting that PPARγ activation enhances NO production and protects against vascular injury.[7,8] Activation of PPARγ also inhibits 2 other processes critical for vascular lesion formation, cell proliferation, and migration.[3,5,6,9,10] In vivo, 2 TZDs, troglitazone (TRO) and pioglitazone, significantly reduced arterial neointimal hyperplasia after endothelial injury in rats.[11–13] In such balloon-catheterized arteries, neointima formation essentially reflects increased migration and proliferation of VSMCs, a major contributor to the growth of atherosclerotic lesions. TRO also inhibited neointima formation in stents placed in the coronary arteries of patients with type 2 diabetes.[14]

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We and others have recently demonstrated that PPARγ activation by TZDs and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibits EC expression of vascular cell adhesion molecule-1, which mediates monocyte adherence to the endothelial surface.[4,15] Because inflammation, dysregulated growth, and migration of monocytes and VSMCs play an important role in the development of atherosclerosis, we hypothesized that PPARγ activation in cells of the vasculature would inhibit the atherosclerotic process. On the other hand, TZDs also stimulate conversion of macrophages into foam cells; therefore, ligand-dependent activation of PPARγ has been postulated to promote atherosclerosis.[16]

Received August 10, 2000; revision accepted December 1, 2000.

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The impact of TZDs on atherosclerosis is a critical issue. TZDs improve insulin-mediated glucose uptake and are used extensively in the treatment of insulin resistance and type 2 diabetes mellitus.[17] Coronary artery disease mortality is increased 2- to 4-fold in type 2 diabetes.[18] Atherosclerosis is the major cause of demise in people with diabetes; therefore, it is important to determine the action of any antidiabetic drug on the atherosclerotic process.

To determine whether PPARγ activation has proatherogenic or antiatherogenic effects, we administered TRO to male LDL receptor–deficient ($LDLR^{-/-}$) mice fed either a high-fat or a high-fructose atherogenic diet. Both models develop substantial hypercholesterolemia and macrophage-laden lesions, designated intimal xanthomata, which do not normally progress to mature atherosclerotic plaques.[19] In addition, the high-fat diet induces hyperglycemia and hyperinsulinemia in the $LDLR^{-/-}$ mouse, making it also a model of type 2 diabetes.[20,21] In contrast, fructose does not increase glucose or insulin in this model[21] and, therefore, was useful because the effects of TZDs on atherosclerosis could be studied in the absence of improvements in insulin action.

Methods

Transendothelial Monocyte Migration

THP-1 cells (5×10^4), a human monocytic leukemia cell line, were added to a human aortic EC monolayer covering a gelatin-coated 8-μm porous membrane and incubated for 30 minutes at 37°C to facilitate their attachment. Cells were then pretreated with the indicated ligands or vehicle (dimethyl sulfoxide) for 30 minutes at 37°C. Migration was induced by the addition of monocyte chemoattractant protein-1 (MCP-1, 50 ng/mL) to the lower compartment. After 90 minutes, nonmigrating THP-1 cells and human aortic ECs were removed with a cotton tip, and the membranes were fixed and stained with the Quik-Diff Stain Set (DADE, Miami, Fla) to identify migrated cells. The number of migrated cells was determined per ×320 high-power field. Experiments were performed in duplicate and were repeated at least 3 times.

Western Blots

Western immunoblots were performed as previously described.[10] Membranes were incubated with rabbit polyclonal antibodies (1:1000 dilution, New England Biolabs) that recognize either (1) total extracellular signal–regulated kinase (ERK) or (2) ERK phosphorylated on threonine 202 and tyrosine 204.

Animals and Diets

Male $LDLR^{-/-}$ mice were obtained (C57BL/6J-$Ldlr^{tm1Her}$, stock No. 002207, Jackson Laboratory, Bar Harbor, Me) and were group-housed under a 12-hour light and 12-hour dark regimen. All animal protocols were approved by the UCLA Animal Research Committee and complied with all federal, state, and institutional regulations. At 3 months of age, the mice were randomly assigned to 1 of 5 dietary regimens: (1) chow (Harlan Teklad 8604), (2) high-fat complex carbohydrate (Research Diets), (3) high-fat complex carbohydrate with 4 g TRO/kg of food, (4) high fructose (Research Diets), or (5) high fructose with 4 g TRO/kg of food. The high-fat diet consisted of 21% fat, 20% protein, 50% carbohydrate, and 0.15% cholesterol. Our high-fat diet differed from those commonly used to study atherogenesis in $LDLR^{-/-}$ mice in that the majority of the nonfat energy came from complex carbohydrate sources instead of sucrose. The high-fructose diet contained 4% fat, 16% protein, 71% fructose, and 0.15% cholesterol. Sources of fat in the diets were corn oil (1% in all diets) and anhydrous milk fat (3% in the fructose diets and 20% in the high fat diets). Mice and feed were weighed weekly, and the rate of consumption of drug was computed. The mice were fed for a period of 12 weeks.

Metabolic Measurements

Blood samples from the retro-orbital sinus were obtained from the mice before the beginning of treatment and every month thereafter and from the abdominal vena cava at euthanasia. Mice were fasted overnight before the collection of the blood samples. Plasma glucose was measured by glucose oxidase reaction (Beckman Glucose Analyzer 2, Beckman Instruments). Plasma lipids were measured by the UCLA Lipid Analysis Laboratory. Plasma insulin was determined by ELISA. Blood pressures were obtained by using an indirect tail-cuff method with a controlled temperature chamber (IITC, Inc) by a technician blinded to the treatment groups.

Vessel Preparation and Image Analysis

Mice were euthanized and perfused with 7.5% sucrose in 4% paraformaldehyde. Aortas were dissected out, split longitudinally, pinned flat in a dissection pan, and stained with Sudan IV to detect lipids and determine lesion area. Images were captured by use of a Sony 3-CCD video camera and analyzed by a single technician who was blinded to the study protocol and used ImagePro image analysis software. The extent of lesion formation is expressed as the percentage of the total aortic surface area covered by lesions.

Cross Sections: Determination of Intimal Macrophage Content

The largest lesions from the aortic arch were excised and embedded in paraffin. The avidin-biotin-peroxidase complex technique for immunostaining was used. Macrophages were stained by using monoclonal antibody to CD68 (titer 1:100, KP1 clone, M0814, Dako Corp). Nonimmune serum was used as a control. Primary antibody incubations were performed in 1% BSA/2% goat serum containing PBS for 60 minutes. Biotinylated rabbit anti-mouse (Dako) was applied; incubation with a streptavidin-peroxidase complex followed. Peroxidase activity was detected with the use of diaminobenzidine tetrahydrochloride as a chromogen. Slides were then counterstained with hematoxylin. Images of the stained sections were analyzed by using the software described above. After tracing the intimal area to be measured with a cursor, 5 pixels of color, which defined the anti-CD68 stain, were sampled by the operator. The area encompassed by the pixels, which was not contiguous, in the color range for anti-CD68 was then computed automatically by the software. This approach has been successfully used by Shi et al[22] to quantify lesional macrophages in a mouse model of transplant arteriosclerosis.

Statistical Analysis

Statistical analysis was performed by using 2-factorial ANOVA with Student-Newman-Keuls to determine the differences between individual group means.

Results

TRO Inhibits Monocyte Migration

VSMC migration and proliferation play an important atherogenic role in the progression of fatty streaks toward more advanced atherosclerotic lesions, such as transitional lesions and classical atheromas. We have previously shown that PPARγ ligands inhibit ERK mitogen-activated protein kinase (MAPK)-dependent migration of VSMCs.[10,11] However, in the earliest stages of atherosclerotic lesions, recruitment of adherent monocytes through their migration into the subendothelium and their phenotypic transformation to macrophages and foam cells play a far greater role than VSMCs in humans and in murine models.[23]

To investigate whether TRO-mediated PPARγ activation affects monocyte recruitment and to further explore its mechanism, we carried out a series of in vitro experiments before our in vivo studies. MCP-1 is an important in vivo migration factor promoting the subendothelial accumulation of monocytes. TRO inhibited MCP-1–directed transmigra-

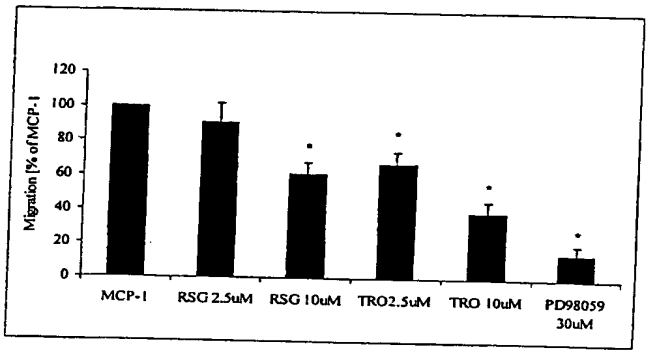

Figure 1. PPARγ ligands inhibit MCP-1–directed transendothelial migration of monocytes. Migration of THP-1 monocytes through ECs was determined by using a modified Boyden chamber assay as described in Methods. The number of migrating cells was quantified by microscopy with the use of high-power fields. Results represent 3 independent experiments performed in duplicate. *$P<0.05$ vs MCP-1 alone.

tion of THP-1 monocytes by 32.7±6.5% at 2.5 μmol/L and by 61.4±6.7% at 10 μmol/L (Figure 1). TRO contains a vitamin E moiety that may confer an antioxidant activity that can inhibit monocyte recruitment and endothelial expression of adhesion molecules. However, rosiglitazone (RSG), another PPARγ ligand that lacks antioxidant activity, also inhibited monocyte transmigration, albeit with a lesser potency than TRO (Figure 1). Inhibition of monocyte transmigration by TRO, therefore, is likely to be mediated at least in part through PPARγ.

MCP-1 rapidly induced ERK activation, reaching a peak at 5 minutes, which was blocked by PD98059, an inhibitor of MAPK ERK kinase (MEK, an upstream kinase), which phosphorylates and activates ERK (Figure 2). PD98059 attenuated MCP-1–directed transmigration by 84.8±4.8%. In combination, these data suggest that activation of PPARγ in monocytes may inhibit their migration by interfering with ERK-MAPK signaling, although the precise mechanism remains to be determined.

Figure 2. MCP-1 activates the ERK-MAPK pathway in THP-1 human monocytes. A, Quiescent (Q) THP-1 cells were stimulated with MCP-1 (50 ng/mL) for 5 minutes. Whole-cell protein extracts were immunoblotted with a phosphospecific ERK1 (pERK1)/ERK2 (pERK2) MAPK antibody. A representative blot of 3 different experiments is shown. B, Conditions were the same as in panel A except that cells were treated with MEK inhibitor PD98059 (1 to 30 μmol/L) or vehicle (dimethyl sulfoxide, –) before and during stimulation with MCP-1 (50 ng/mL). A representative blot of 3 different experiments is shown.

TRO Inhibits Intimal Macrophage Accumulation and Lesion Formation in Male LDLR$^{-/-}$ Mice

LDLR$^{-/-}$ mice that were fed a regular chow diet develop few lesions across the surface of the aorta. Male 3-month-old LDLR$^{-/-}$ mice were placed on either a high-fat or high-fructose diet to induce atherosclerosis. LDLR$^{-/-}$ males were used in the present study because they develop hyperglycemia and become diabetic on a high-fat diet but remain normoglycemic when fed a high-fructose diet. Moreover, males develop twice the level of surface lesions as do females,[24] and their use obviates the potentially confounding influence of the vascular protection in females afforded by estrogen. Comparison of the impact of TRO on atherogenesis in these 2 dietary models was undertaken to distinguish any activity of PPARγ to normalize metabolic abnormalities accompanying diabetes that contribute to high-fat–induced xanthomata formation from any direct effects on the vasculature. To assess the impact of TRO on aortic lesions, 1 high-fat diet group and 1 high-fructose diet group received TRO at 400 mg/kg body wt per day from drugs pelleted into the atherogenic diets. This dose of TRO was chosen because we previously demonstrated its efficacy in inhibiting intimal hyperplasia in rats after balloon injury.[11]

The en face method, which makes use of computer-assisted analysis of color images of Sudan IV–stained lipid-containing material in the entire aorta, was used to determine the percentage of surface area affected by lesions.[24] Male LDLR$^{-/-}$ mice on normal chow for 3 months had <0.20% lesions (Figure 3A). The high-fat diet increased the amount of surface lesions after 3 months to 3.90±0.16% (n=8, Figure 3B). TRO inhibited the high-fat–induced lesions by 30% (2.76±0.36% of the aortic surface, n=8, $P<0.02$; Figure 3C). Similar to Merat et al,[21] we noted that the high-fructose diet was more atherogenic than the high-fat diet, causing 8.42±0.94% lesions (n=17, Figure 3D). TRO reduced lesions in fructose-fed LDLR$^{-/-}$ males by 42% (4.90±0.65%, n=14, $P<0.01$; Figure 3E). Quantitative results are summarized in Figure 4.

TRO-treated male LDLR$^{-/-}$ mice fed either the high-fat or high-fructose diet for 3 months developed lesions that contained substantially fewer CD68-staining macrophages (Figure 5A through 5D). Lesions induced by a high-fat diet contained 39.1±6.8% macrophages (percent of cross-sectional intimal area) compared with 13.3±4.9% ($P<0.01$) in mice administered TRO (Figure 5E). Similar results were obtained for males fed the high-fructose diet, where TRO decreased macrophage accumulation from 40.4±3.5% to 17.1±1.7% ($P<0.01$, Figure 5E). The lesions in the TRO-fed animals tended to be smaller in volume than those in males not fed TRO. The relative macrophage content in the larger lesions (not treated with TRO) exceeded the content in the smaller lesions (treated with TRO) by 140% to 200%. The reduction in macrophage accumulation in the lesions of TRO-treated animals is unlikely to be the result of their being an earlier lesion stage, because the relative macrophage content is known to be greatest in the smaller (ie, early-stage) lesions.

Effect of TRO on Metabolic Parameters

All metabolic measurements determined on blood samples drawn before treatment were similar in all groups (Tables 1

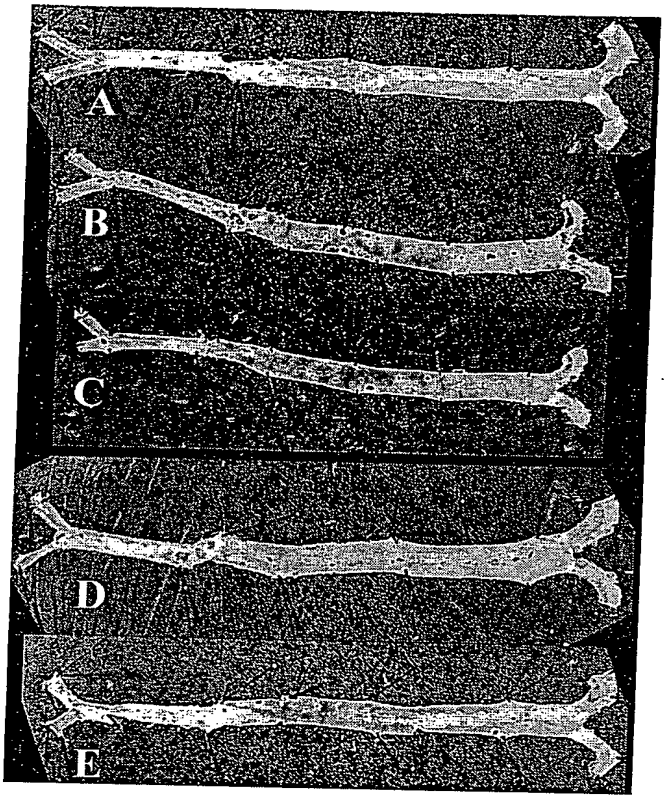

Figure 3. TRO attenuates atherosclerosis in male $LDLR^{-/-}$ mice. The aorta is stained by Sudan IV to detect the lipids present in lesions. A, Chow diet. B, High-fat diet. C, High-fat diet and TRO. D, High-fructose diet. E, High-fructose diet and TRO.

and 2). In accordance with previous studies on male $LDLR^{-/-}$ mice, we found that a high-fat diet induced diabetes[20,21] (Table 1). Glucose levels progressively increased throughout

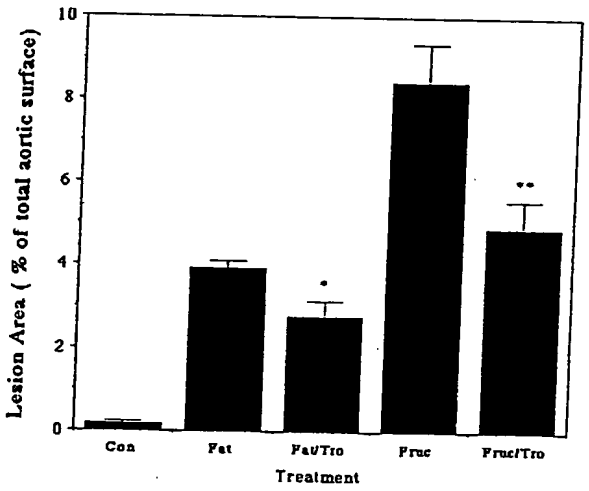

Figure 4. Quantification of the antiatherogenic activity of TRO in male $LDLR^{-/-}$ mice. Mean atherosclerotic surface lesion areas were determined in mice fed a normal chow, high-fat, or high-fructose diet in the absence or presence of TRO for 3 months. Image analysis and quantification of the percentage of the total aortic area staining for Sudan IV were performed by using computer-assisted image analysis. TRO produced a significant decrease in mice fed a high-fat (30% decrease, $^{*}P<0.05$) and high-fructose (42% decrease, $^{**}P<0.05$) diet.

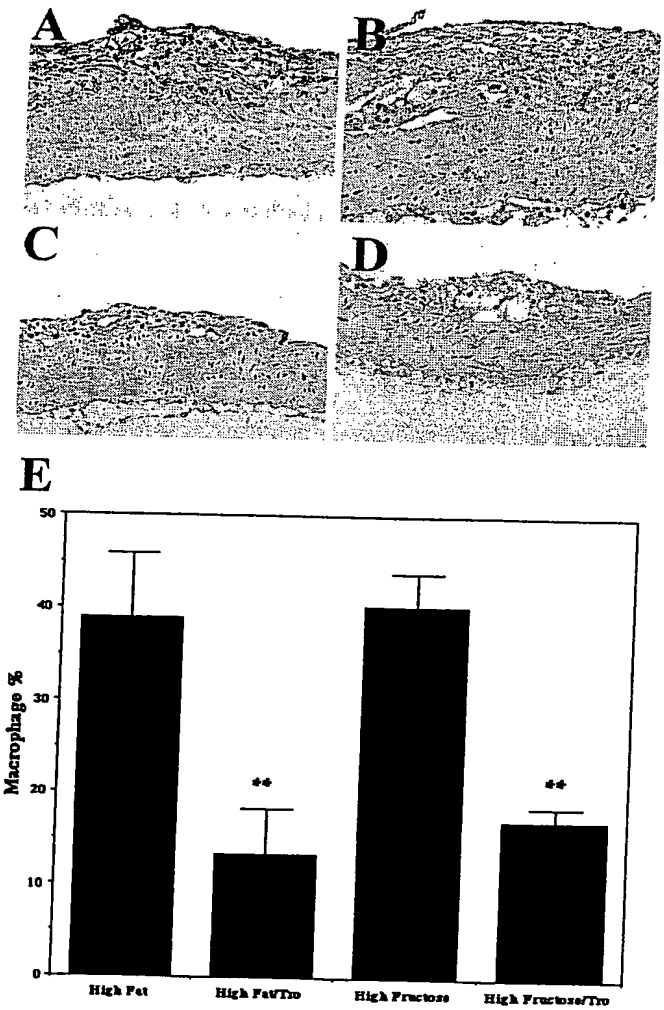

Figure 5. TRO inhibits accumulation of lesional macrophages. Sections from the aortic arch were immunostained by using antibody against CD68 to detect macrophages. Quantification of the percentage of the intimal area staining ($^{**}P<0.05$) for CD68 was performed by computer-assisted image analysis. A, High-fat diet (n=6). B, High-fat diet and TRO (n=6). C, High-fructose diet (n=6). D, High-fructose diet and TRO (n=6). E, Quantification of the macrophage content.

the study, reaching a maximum of 285 mg/dL at 3 months compared with 148 mg/dL for mice on normal chow. The fat-fed males were also hyperinsulinemic (1198±149 versus 664±113 pg/mL on normal chow), consistent with the development of early-stage type II diabetes. Although TRO did not decrease hyperglycemia in high-fat–fed male mice, TRO administration completely normalized their plasma insulin levels. In marked contrast, mice on a high-fructose diet had normal fasting plasma glucose and insulin levels, which were not altered by TRO.

$LDLR^{-/-}$ males developed severe hypercholesterolemia on either the high-fat or high-fructose diet, achieving levels 3- to 4-fold greater than those in animals maintained on regular chow (Table 2). TRO lowered total plasma cholesterol by 27% in males on the high-fructose diet but had no effect on the high-fat–fed mice. Triglycerides were elevated in the high-fat–fed males but not in the high-fructose group; TRO

TABLE 1. Plasma Glucose and Insulin Levels and Final Body Weights

	Chow Diet	High-Fat Diet	High-Fat Diet/TRO	High-Fructose Diet	High-Fructose Diet/TRO
Glucose, mg/dL					
Start	158.7±14.63	173.2±12.12	169.4±7.98	137.8±11.53	148.5±27.67
1 mo	148.9±6.60	190.5±10.92	155.1±9.13*	121.2±6.44	114.8±4.55
2 mo	144.8±10.96	201.1±9.09	189.6±27.37	128.6±13.09	121.89±17.32
3 mo	148.5±14.87	284.8±25.00	268.9±13.90	152.3±15.80	181.9±23.47
Insulin, pg/mL					
3 mo	664.7±113.62	1198.7±149.81	691.2±109.14†	304.4±47.49	278.7±37.9
Body weight, g	27.3±0.56	42.5±0.66	37.0±0.89†	25.9±0.37	24.2±0.34

Values are mean±SEM.
*$P<0.05$ vs high-fat; †$P<0.01$ vs high-fat diet.

did not alter triglycerides in either model. HDL cholesterol (HDLC) decreased with both of the diets, compared with normal chow, as frequently reported.[3] TRO further lowered the HDLC in the high-fat–fed males but increased it in the high-fructose–fed group. Plasma free fatty acid levels increased in males on the high-fat diet but not in those on the high-fructose diet; TRO decreased free fatty acid levels in both models.

Discussion

The most significant finding of the present study is that TRO inhibited lesion formation in a type 2 diabetic mouse model and a nondiabetic LDLR$^{-/-}$ mouse model of intimal xanthomata. Mice fed the high-fat diet developed extensive hypercholesterolemia that was not affected by TRO. These mice also gained substantial weight and showed an increase in circulating free fatty acid levels, which probably contributed to their insulin resistance, hyperinsulinemia, and fasting hyperglycemia.[25] The increase in triglycerides and decrease in HDLC are consistent with insulin resistance. TRO decreased circulating insulin but did not affect glucose in this model. The same has been reported in humans with type 2 diabetes, of whom 20% treated with TRO showed no improvement in glucose control, but all demonstrated improved insulin sensitivity.[26] In contrast to the response in humans, TRO did not alter triglycerides and further decreased HDLC. Mice fed the high-fructose diet also developed severe hypercholesterolemia but did not gain weight or develop hyperinsulinemia or elevations in free fatty acids or triglycerides. In this model, TRO decreased the free fatty acids, increased HDLC, and decreased total cholesterol.

TABLE 2. Plasma Lipid Levels

	Chow Diet	High-Fat Diet	High-Fat Diet/TRO	High-Fructose Diet	High-Fructose Diet/TRO
Total Cholesterol, mg/dL					
Start	292.2±14.63	277.7±5.77	278.8±9.24	321.8±20.87	328.0±18.32
1 mo	316.0±11.51	583.3±72.18	541.9±62.22	489.1±24.58	360.8±21.37†
2 mo	315.8±10.26	1307.0±110.11	1173.0±122.11	1052.3±33.78	816.7±25.02‡
3 mo/final	317.9±17.79	1341.9±52.14	1313.63±28.83	1167.7±46.17	862.1±23.70‡
HDLC, mg/dL					
Start	110.6±4.51	111.2±1.87	109.9±3.07	121.2±2.59	113.5±8.39
1 mo	111.2±3.19	108.2±1.93	108.9±2.65	104.2±3.08	106.6±4.58
2 mo	112.1±4.06	94.4±3.52	98.2±12.66	100.4±3.56	105.3±4.76
3 mo	112.4±5.00	104.8±7.84	81.8±6.86*	90.1±4.48	108.4±5.10†
Free fatty acids, mg/dL					
Start	67.1±2.82	63.5±1.82	58.2±2.67	91.0±6.96	82.5±4.38
1 mo	69.4±2.97	70.4±2.11	66.6±2.67	66.9±4.90	71.2±3.75
2 mo	65.7±2.87	88.6±7.32	74.2±4.19	68.7±3.67	65.8±3.57
3 mo	61.7±3.68	72.5±2.42	57.1±2.07*	61.7±1.52	53.1±2.81†
Triglycerides, mg/dL					
Start	122.0±4.43	109.1±5.32	101.1±5.72	84.8±5.09	111.83±16.00
1 mo	126.0±11.1	124.3±7.00	113.6±7.48	85.8±4.98	94.0±6.47
2 mo	86.9±4.98	156.6±30.55	191.8±64.47	81.2±5.98	79.8±6.47
3 mo	71.6±6.92	141.8±7.84	159.1±19.79	75.3±6.89	69.0±4.85

Values are mean±SEM.
*$P<0.01$ vs high-fat diet; †$P<0.05$ vs high-fructose diet, and ‡$P<0.001$ vs high-fructose diet.

Despite the difference in metabolic responses between the diabetic and nondiabetic animals, both hypercholesterolemic models responded to TRO with decreased lesion formation. These results suggest that TRO has direct vascular effects, separate from its metabolic effects, that decrease the atherosclerotic process. Alternatively, the antiatherogenic effects of TRO in the 2 different models might involve the collection of distinct metabolic processes. For example, hemodynamic effects of TRO related to its reported activity to lower blood pressure in animal models and in humans could also impact pathophysiological processes in high-fat– and high-fructose–fed LDLR$^{-/-}$ mice.[27–30] All major cell types contributing to this vascular lesion formation express PPARγ, which provides a mechanism for the direct effect of thiazolidinedione ligands in the vessel wall.[3,5,6,9] Data from in vitro experiments had suggested mechanisms by which activation of PPARγ could either accelerate or attenuate the atherosclerotic process.[2–6,9,10,16] The present study provides conclusive evidence that ligand-induced PPARγ activation by TRO reduces intimal xanthomata in murine models.

TRO had several systemic effects that may have contributed to its attenuation of intimal xanthomata. In the diabetic high-fat–fed mouse, TRO lowered insulin and glucose levels and decreased HDLC (which is thought to promote atherogenesis). In the fructose-fed model, TRO decreased total cholesterol and increased HDLC. Our finding that TRO was more potent in suppressing lesion formation in the fructose-fed model compared with the high-fat–fed mice could be due to the observed 27% reduction in total cholesterol. A common effect of TRO in the high-fat–fed and high-fructose–fed LDLR$^{-/-}$ models is its suppression of circulating free fatty acid levels. However, increased circulating free fatty acids have not been shown to be an independent risk factor for atherosclerosis.

Inflammation in the vascular wall has clearly emerged as a major culprit in the development of atherosclerosis.[31] Damage to the endothelium and the subsequent recruitment and transendothelial migration of monocytes constitute critical early cellular responses during atherogenesis.[31] Transmigration of monocytes into the subendothelial space is strongly stimulated by the chemokine MCP-1, which is expressed and secreted by ECs and VSMCs. The essential role of MCP-1 in atherogenesis is underscored by a recent study demonstrating that crossing MCP-1–deficient mice into LDLR$^{-/-}$ mice attenuated lesion formation by >80%.[32] Our group and others have shown that TRO and other PPARγ ligands inhibit growth factor–directed ERK-MAPK–dependent VSMC migration.[5,10,11] Cell migration requires de novo gene transcription that is consistent with PPARγ acting in the nucleus to inhibit this process.[10] In particular, activation of PPARγ can inhibit ERK-MAPK signaling to the nucleus.[11,33] Because MCP-1–directed migration of monocytes is ERK-MAPK dependent, interference with this pathway by TRO could contribute to the observed reduction in intimal xanthomata and lesional macrophages in treated LDLR$^{-/-}$ mice.

TRO and another PPARγ ligand, RSG, which does not contain an α-tocopherol moiety, inhibited MCP-1–directed migration of human monocytes in vitro. TRO also consistently decreased intimal macrophage accumulation in the diabetic and nondiabetic mice. These findings support the concept that inhibition of monocyte attachment and migration in the vessel by TRO may be one of the mechanisms contributing to the reduction of atherogenesis. Although it cannot be ruled out that the reduction of intimal monocytes in part reflected the reduced lesion size induced by TRO treatment, this is unlikely to be the sole explanation, because the relative intimal monocyte/macrophage content is known to be greatest in the early stages (smaller lesions) of atherosclerosis. In any case, the antiatherosclerotic activity of TRO-induced PPARγ activation clearly prevailed over its hypothesized promotion of foam cell formation via increased expression of the scavenger receptor CD36.[16]

Unlike other PPARγ ligands, TRO has an α-tocopherol (vitamin E) moiety that theoretically could contribute to its antiatherogenic activity through antioxidant effects.[34] Vitamin E has been shown to suppress atherosclerosis in the apoE knockout model, which develops advanced atherosclerotic lesions.[35,36] Whether the dose of vitamin E provided by TRO in the present study is enough to impact lesion formation is doubtful. At 400 mg/kg TRO per day, LDLR$^{-/-}$ mice received the equivalent of 8 IU of vitamin E, a dose much lower than that reported to affect atherosclerosis or to significantly protect LDL against oxidation.[35–38] Another line of evidence for the assumption that the effect of TRO on lesion formation was not, to a significant degree, dependent on antioxidant effects is provided by a parallel study demonstrating that 2 other PPARγ ligands, RSG and GW7845,which do not contain the α-tocopherol moiety, inhibited atherogenesis in the aortic root of male LDLR$^{-/-}$ mice fed a high-fat, cholesterol-enriched diet.[39] In addition, the recent Heart Outcomes Prevention Evaluation (HOPE) clinical trial in humans did not show an effect of vitamin E on coronary artery disease events or mortality.[40]

In summary, given the absence of consistent major metabolic changes present in diabetic and nondiabetic mice, it is likely that TRO at least in part decreases early atherosclerotic lesion formation through direct vascular effects. In human subjects with diabetes, who have a high risk for coronary disease, TRO improves insulin resistance and other proatherogenic metabolic parameters, which may improve cardiovascular risk. It is possible that some of the vascular effects observed in our murine models may also be present in humans. Although Li et al[39] and our data demonstrate that PPARγ ligands suppress early atherosclerotic lesions, intimal xanthomata do not inexorably progress to more advanced atherosclerotic plaques; in fact, they often regress.[19] Therefore, determining the effects of PPARγ ligands on more advanced atherosclerotic lesions may prove to be a stronger predictor of their potential clinical benefit. Nonetheless, the present results indicate that an investigation of potential antiatherogenic effects of PPARγ ligands is strongly warranted.

Acknowledgments

This work was supported by National Institutes of Health grant HL-58328 to Willa A. Hsueh. Shu Wakino was supported by a Mary K. Iacocca Fellowship in Diabetes. Ulrich Kintscher was supported by a Gonda (Goldschmeid) Fellowship in Diabetes.

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